PCR Primers for Detection of *Pantoea ananatis*, *Burkholderia* spp., and *Enterobacter* sp. from Onion

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Abstract

Asselin, J. E., Bonasera, J. M., and Beer, S. V. 2016. PCR primers for detection of *Pantoea ananatis*, *Burkholderia* spp., and *Enterobacter* sp. from onion. Plant Dis. 100:836-846.

Bacterial decays of onion bulbs cause sporadic and sometimes serious losses to onion (*Allium cepa*). In New York, three groups of bacteria were identified as problematic: *Burkholderia* spp., *Pantoea ananatis*, and *Enterobacter* spp. To aid in efficient detection and diagnosis of these pathogens, pairs of specific polymerase chain reaction primers were designed and validated, based on a strategy that utilized various genome

sequences now available in public databases. Primer pairs were tested against numerous strains of target bacteria, closely related bacteria, and other onion-pathogenic bacteria. Each primer pair yielded a single, apparently highly specific amplicon from aqueous suspensions of the target bacteria. Minimum sensitivities were approximately 10^3 CFU per 25- μ l reaction mixture for all three primer pairs.

Onion (*Allium cepa*) bulbs are susceptible to a number of diseases caused by bacteria. Symptoms can range from slight discoloration of one or a few internal scales to severe maceration of almost the entire bulb (Schwartz and Mohan 2008). Losses are a continuing and sometimes severe problem for onion growers. Symptom development can occur during the growing season or during harvest, curing, and cold storage of bulbs prior to marketing. Different bacterial pathogens cause different symptoms in onion bulbs. However, symptoms are not sufficiently distinct to serve as identifiers of a causal organism, especially for bulbs with advanced disease. Mixed infections and the presence of secondary invaders are common and can create confounding combinations of symptoms. In New York State, several bacterial pathogens of onion have been identified, including *Pantoea ananatis* (Carr et al. 2010) and species of *Burkholderia* (Burkholder 1950) and *Enterobacter* (Zaid et al. 2011) (hereinafter target bacteria).

Several species of *Burkholderia* (*Burkholderia gladioli*, *B. cepacia*, and *B. cenocepacia*) have been described previously as onion pathogens (Burkholder 1942, 1950; Sotokawa and Takikawa 2004). Onion diseases caused by *Burkholderia* spp. are characterized by water soaking and maceration of infected bulb scales. *B. cepacia* and *B. cenocepacia* belong to the *Burkholderia cepacia* complex (BCC), a group of at least 20 closely related species within the genus *Burkholderia* (De Smet et al. 2015; Peeters et al. 2013; Vandamme and Peeters 2014). BCC bacteria and *B. gladioli* are also opportunistic human pathogens, causing serious infections in individuals with cystic fibrosis or chronic granulomatous disease. As such, polymerase chain reaction (PCR)-based tools to identify these strains were developed previously to aid in diagnosis of clinical infections. These tools include primers that produce amplicons from strains of the BCC or broadly from the genus *Burkholderia* (Mahenthiralingam et al. 2000; Payne et al. 2005). Primers have also been reported for detecting particular species of the BCC or *B. gladioli* (Mahenthiralingam et al. 2000; Whitby et al. 2000). However, the continued delineation of species within

the BCC means that PCR and other protocols identifying strains to the species level must be revalidated as changes in taxonomy are made.

P. ananatis is a pathogen of several food crops and causes center rot of onion. Symptoms of *P. ananatis* infection in onion plants include lesions on the leaves and a yellow-brown discoloration of bulb scales in infected bulbs. Similar symptoms can be caused by pathogenic strains of the related species *P. agglomerans* (Hattingh and Walters 1981) and *P. allii* (Brady et al. 2011). Several primer sets have been published previously (Carr et al. 2010; Figueiredo and Paccola-Meirelles 2012; Gitaitis et al. 2002). All are based on detection of the multiple copy 16S/23S ribosomal internal transcribed spacer (ITS) regions. In addition to *P. ananatis*, these primers also reportedly amplify DNA of at least one strain of *P. stewartii* (Gitaitis et al. 2002) or strains of *P. allii* (Figueiredo and Paccola-Meirelles 2012).

The symptoms of *Enterobacter* bulb decay of onion are usually mild, with discolored internal scales and no maceration. The disease is usually noted as a problem in cold storage (Schwartz and Otto 2000; Zaid et al. 2011) but it has been described as a late-season disease in the field following periods of excessive heat (Bishop and Davis 1990; Schroeder and du Toit 2009). Recent work suggested that the species of *Enterobacter* most commonly isolated from onion in Washington may not be *Enterobacter cloacae* but, perhaps, another species of *Enterobacter* (B. Schroeder, personal communication). These strains appear to be pathogenic to onion in inoculation assays (B. Schroeder, personal communication). We are not aware of any diagnostic PCR primer pairs that amplify DNA from *Enterobacter* spp. generally, or the group of strains most commonly isolated from onion specifically.

In order to study the prevalence, timing, and sources of inoculum of bacterial pathogens of onion bulbs, accurate techniques for detecting decay pathogens are crucial. Pathogen-specific PCR primers producing single amplicons with genomic DNA from target bacteria are valuable for quickly and inexpensively detecting bacteria from a mixture or a pure culture. Here, we report the design and validation of three specific primer pairs for detection of the three important groups of bacteria that infect onion in New York. Each primer set is specific to a particular subset of bacteria: *P. ananatis*, BCC and *B. gladioli*, and the group of *Enterobacter* strains most commonly isolated from onion.

Our primer design strategy used genomic data in GenBank and other public databases to identify DNA sequences common to multiple strains of the bacteria of interest but absent from other genome sequences of bacteria. PCR primer pairs based on those sequences

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*The e-Xtra logo stands for "electronic extra" and indicates that two supplementary figures are published online.

Accepted for publication 21 October 2015.

<http://dx.doi.org/10.1094/PDIS-08-15-0941-RE>
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were designed and tested empirically using numerous target and non-target bacterial strains.

Materials and Methods

Bacterial strains used in this study. Bacterial strains were routinely cultured on Luria-Bertani (LB) agar plates and incubated at

28 or 37°C for 1 to 2 days. For long-term storage, freshly grown bacterial strains were lifted from LB agar plates using sterile cotton-tipped applicators, suspended in 15% aqueous glycerol, and stored at –80°C. Separate sets of strains were used for assessing the specificity of primers designed to detect *Burkholderia* spp. (Table 1), *P. ananatis* (Table 2), and *Enterobacter* spp. (Table 3) toward target

Table 1. Strains used to test *Burkholderia*-specific primers

Strain	Isolated from	Other number	Received from or reference	Amp ^a
BCC^b				
CU0318	Onion	64-22NS	Zaid et al. (2012)	+
CU3094	Soil, New Jersey			+
CU3368	Onion, 1948	ATCC 25416	Type strain of <i>Burkholderia cepacia</i>	+
CU3370	Soil, New Jersey	ATCC 39356		+
CU6878	Onion field soil, New York	Christy IV	Zaid et al. (2012)	+
CU3372	Forest soil, Trinidad	ATCC 17759		+
CU3371	Corn field soil, New Jersey	ATCC 39277		+
D21	Onion, New York		S. V. Beer	+
E40b	Onion, New York		S. V. Beer	+
F37b	Onion, New York		S. V. Beer	+
F40a	Onion, New York		S. V. Beer	+
I49a	Onion, New York		S. V. Beer	+
I55b	Onion, New York		S. V. Beer	+
J20a	Onion, New York		S. V. Beer	+
J31	Onion, New York		S. V. Beer	+
J32a	Onion, New York		S. V. Beer	+
J74a	Onion, New York		S. V. Beer	+
J81b	Onion, New York		S. V. Beer	+
J83a	Onion, New York		S. V. Beer	+
J96c	Onion, New York		S. V. Beer	+
K21b	Onion, New York		S. V. Beer	+
K37C	Onion, New York		S. V. Beer	+
K4b	Onion, New York		S. V. Beer	+
L15a	Onion, New York		S. V. Beer	+
L24b	Onion, New York		S. V. Beer	+
L88a	Onion, New York		S. V. Beer	+
M17b	Onion, New York		S. V. Beer	+
M18a	Onion, New York		S. V. Beer	+
M2	Onion, New York		S. V. Beer	+
<i>B. gladioli</i>				
CU3082	Onion, New York		W. H. Burkholder	+
CU3083	Onion, New York	PA-23	Yohalem and Lorbeer (1994)	+
CU3890		ATCC 10248		+
CU3891	Onion bulb rot	ATCC 19302		+
Similar to <i>B. phymatum</i>				
H49a	Onion, New York		S. V. Beer	–
I44a	Onion, New York		S. V. Beer	–
I49b	Onion, New York		S. V. Beer	–
I54b	Onion, New York		S. V. Beer	–
K37B	Onion, New York		S. V. Beer	–
K5b	Onion, New York		S. V. Beer	–
L52a	Onion, New York		S. V. Beer	–
P19a	Onion, New York		S. V. Beer	–
K3b	Onion, New York		S. V. Beer	–
<i>Burkholderia</i> spp.				
O64a	Onion, New York		S. V. Beer	–
Other genera				
<i>Pseudomonas protegens</i>				
A60	Onion, New York		S. V. Beer	–
H10a	Onion, New York		S. V. Beer	–
<i>Pseudomonas putida</i>				
E65	Onion, New York		S. V. Beer	–
<i>Pseudomonas</i> spp.				
A1	Onion, New York		S. V. Beer	–
H40c	Onion, New York		S. V. Beer	–
<i>Xanthomonas axonopodis</i> pv. <i>allii</i>				
CU6923	Onion, CO	O274	H. Schwartz, CSU ^c	–

^a Amplification with BAMB_5978 40F/350R.

^b *Burkholderia cepacia* complex.

^c CSU = Colorado State University.

Table 2. Strains used to test *Pantoea ananatis*-specific primers

Strain	Isolated from	Other number	Received from or reference ^a	Amp ^b
<i>P. agglomerans</i>				
Eh220	Onion, South Africa.	LMG 2595 SUH 1	M. J. Hattingh; Hattingh and Walters (1981)	–
Eh221	Onion, South Africa.	LMG 2596 SUH 2	M. J. Hattingh; Hattingh and Walters (1981)	–
Eh222	Onion, South Africa.	SUH 4	M. J. Hattingh; Hattingh and Walters (1981).	–
ATCC 27155	Knee laceration		Type strain of <i>P. agglomerans</i>	–
B46	Onion, New York		S. V. Beer	–
E37a	Onion, New York		S. V. Beer	–
J14b	Onion, New York		S. V. Beer	–
<i>P. allii</i>				
LMG24202	Onion, Georgia	BD 309; subculture of Hort. Hill 24	BCCM; Goszczynska et al. (2006a) and Brady et al. (2011)	–
LMG24203	Onion, South Africa.	BD 377	BCCM; Brady et al. (2011)	–
LMG24248	Onion, South Africa.	BD 390	BCCM; Goszczynska et al. (2006a) and (Brady et al. 2011)	–
J12b	Onion, New York		S. V. Beer	–
O64c	Onion, New York		S. V. Beer	–
S37a	Onion field soil, New York		S. V. Beer	–
<i>P. ananatis</i>				
LMG20103	Eucalyptus, South Africa		BCCM	+
LMG24190	Onion seed, South Africa		BCCM	+
LMG24193	Onion seed, South Africa		BCCM	+
LMG2665 (type)	Pineapple, Brazil.	ATCC 33244	BCCM	+
LMG2668	Pineapple, Hawaii		BCCM	+
OC34b	Onion, New York		Carr et al. (2013)	+
OC37	Onion, New York		Carr et al. (2013)	+
OC42	Onion, New York		Carr et al. (2013)	+
OC5a	Onion, New York		Carr et al. (2013)	+
B51	Onion, New York		S. V. Beer	+
D72	Onion, New York		S. V. Beer	+
I53a	Onion, New York		S. V. Beer	+
J21b	Onion, New York		S. V. Beer	+
J32b	Onion, New York		S. V. Beer	+
J63b	Onion, New York		S. V. Beer	+
J78b	Onion, New York		S. V. Beer	+
L32c	Onion, New York		S. V. Beer	+
M46	Onion, New York		S. V. Beer	+
M8c	Onion, New York		S. V. Beer	+
N5	Onion, New York		S. V. Beer	+
N8b	Onion, New York		S. V. Beer	+
O81b	Onion, New York		S. V. Beer	+
R36b	Onion, New York		S. V. Beer	+
T29	Onion, New York		S. V. Beer	+
T53c	Onion, New York		S. V. Beer	+
T85b	Onion, New York		S. V. Beer	+
Pna 97-1	Onion, Georgia		R. Gitaitis; Gitaitis et al. (2002)	+
A8	Melon, Japan		Kido et al. (2010)	+
AJiSAi-9701	Hydrangea, Japan		Kido et al. (2010)	+
CTB1004	Rice, Japan		Kido et al. (2010)	+
PA-5	Pineapple, Japan		Kido et al. (2010)	+
SUPP2219	Rice, Japan		Kido et al. (2010)	+
SWEETCORN-101	Corn, Japan		Kido et al. (2010)	+
Blackshank 15	Onion, Georgia		R. Walcott; Goszczynska et al. (2006b)	+
Hort. Hill 31	Onion, Georgia		R. Walcott; Goszczynska et al. (2006b)	+
BD 622	Corn, South Africa.		Brady et al. (2008)	+
<i>P. stewartii</i> subsp. <i>stewartii</i>				
DC283			D. Coplin; Dolph et al. (1988)	–
Other <i>Pantoea</i>				
Eh282	Pineapple, Hawaii	M189	A. M. Alvarez, UH	–
L4	Onion, New York		S. V. Beer	–
L52b	Onion, New York		S. V. Beer	–
J49a	Onion, New York		S. V. Beer	–

^a BCCM = BCCM/LMG Bacteria Collection and UH = University of Hawaii.^b Amplification with PANA_1080 61F/1009R.

strains and close relatives. An additional set of bacterial strains (Table 4) was used to test the specificity of each primer set against other genera of onion-pathogenic and onion-associated bacteria. A subset of these bacterial pathogens of onion (Table 4) was used to test the performance of the three primer pairs in mixtures of target and nontarget bacteria.

Identification of bacterial strains. Bacterial strains recovered from onion bulbs or onion fields in New York State were identified by amplifying and sequencing a portion of the conserved *gyrB* gene from pure cultures, as described by Bonasera et al. (2014). Amplicons were purified for sequencing using the Clean and Concentrator-5 kit (Zymo Research Corp., Irvine, CA) and sequenced at the Cornell University Biotechnology Resource Center. Sequencing

results were compared with the National Center for Biotechnology Information (NCBI) nucleotide database using blastn. Strains with high percentages of sequence identity to strains identified as *P. ananatis*, *Burkholderia* spp., or *Enterobacter* spp. were of interest. For these, *gyrB* sequences were aligned with sequences from GenBank in the program MegAlign (DNASStar, Madison, WI) using the ClustalW method and visualized as phylogenetic trees in MegAlign. For *Enterobacter* and *Pantoea* spp., the *gyrB* sequences were sufficiently informative to resolve distinct clades for *P. ananatis*, *P. allii*, and *P. agglomerans* as well as a distinct clade for most *Enterobacter* strains isolated from onion. The clade harboring most *Enterobacter* strains isolated from onion included EcWSU1 (CP002886.1), a genome-sequenced strain isolated from onion in Colorado, and

Table 3. Strains used to test *Enterobacter* sp. primers

Strain	Isolated from	Other number	Received from or reference ^a	Amp ^b
Clustering^c				
EcWSU1	Onion, Colorado; subculture of O310		Humann et al. (2011); Schroeder et al. (2010)	+
1.1 WA	Onion	1.1	B. Schroeder; WSU	+
3.3 WA	Onion	3.3	B. Schroeder; WSU	+
4.4 WA	Onion	4.4	B. Schroeder; WSU	+
5.5 WA	Onion	5.5	B. Schroeder; WSU	+
10.6 WA	Onion	10.6	B. Schroeder; WSU	+
11.5 WA	Onion	11.5	B. Schroeder; WSU	+
O121 CO	Colorado Pathogenic to onion.	O121	H. Schwartz; CSU	+
O144 CO	Colorado Pathogenic to onion.	O144	H. Schwartz; CSU	+
O174 CO	Infected onion in field. Colorado	O174	H. Schwartz; CSU	+
O280 CO		O280	H. Schwartz; CSU	+
O310 CO	Onion, Colorado	O310	H. Schwartz; CSU	+
O325 CO	Colorado Pathogenic to onion.	O325	H. Schwartz; CSU	+
Ecl1 NY	Onion, New York	Ecl1	Ali Zaid	+
Ecl2 NY	Onion, New York	Ecl2	Zaid et al. (2012)	+
Ecl3 NY	Soil from onion field, New York	Ecl3; ATCC BAA-2271	Zaid et al. (2011); Zaid et al. (2012)	+
Ecl6 NY	Onion, New York	Ecl6; ATCC BAA-2272	Zaid et al. (2011)	+
A5b	Onion, New York		S. V. Beer	+
A56a	Onion, New York		S. V. Beer	+
A68b	Onion, New York		S. V. Beer	+
F38a	Onion, New York		S. V. Beer	+
H29c	Onion, New York		S. V. Beer	+
I52a	Onion, New York		S. V. Beer	+
J88	Onion, New York		S. V. Beer	+
K64	Onion, New York		S. V. Beer	+
L87a	Onion, New York		S. V. Beer	+
Not clustering^d				
A10a	Onion, New York		S. V. Beer	–
Ecl4 NY	Onion, New York	Ecl4	S. V. Beer	–
Ecl-1 GA	Onion, Georgia	Ecl-1	R. Gitaitis, UG	–
Ecl-2 GA	Onion, Georgia	Ecl-2	R. Gitaitis, UG	–
Ecl-3 GA	Onion, Georgia	Ecl-3	R. Gitaitis, UG	–
Ecl7 NY	Onion field soil, New York	Ecl7; ATCC BAA-2273	Zaid et al. (2011)	–
FC081213-33	Creek adjacent to onion field, New York		S. V. Beer	–
M29c	Onion, New York		S. V. Beer	–
<i>Enterobacter</i> spp.^e				
Ecl 204/77			CDC; Zaid et al. (2012)	–
Ecl 280/77			CDC; Zaid et al. (2012)	–
Ecl ATCC 23355			B. Schroeder, WSU; Zaid et al. (2012)	–
Ecl ATCC 13047	Isolated from spinal fluid; <i>E. cloacae</i> type strain		B. Schroeder, WSU.	–
ATCC 29941			Zaid et al. (2012)	–
Other species				
<i>Klebsiella mobilis</i> 819/56			CDC	–
<i>K. pneumoniae</i> ATCC 15574	Germinating seedlings of <i>Psychotria bacteriophila</i>			–

^a WSU = Washington State University, CSU = Colorado State University, CDC = Centers for Disease Control, and UG = University of Georgia.

^b Amplification with EcWSU1_01990 30F/443R.

^c Strains clustering with EcWSU1–*Enterobacter ludwigii* group in partial *gyrB* tree.

^d *Enterobacter*-like strains from onion or onion fields not clustering with EcWSU1 in partial *gyrB* tree.

^e *Enterobacter* spp. from other sources.

E. ludwigii EN-119 (JTLO01000001.1) (Li et al. 2015), the type strain for that species (Supplementary Fig. S1). For *Burkholderia* spp., partial *gyrB* sequences designated strains as *B. gladioli* or within the BCC. However, the partial *gyrB* sequences were unable to sufficiently differentiate BCC species, because published sequences from strains of the same species did not form distinct clades.

Tests of bacterial strains for pathogenicity to onion bulbs. Several strains identified as *Burkholderia* spp. were assessed for pathogenicity in onion bulbs. Prior to pathogenicity testing, strains were assessed for their ability to grow at 37°C. Strains similar to *B. phymatum* did not grow well at 37°C but did grow well at 28°C. Strain O64a grew well at 37°C. Bacteria were swabbed from LB agar plates into sterile high-purity (HP) water using sterile cotton-tipped applicators. Suspensions were adjusted to an optical density at 600 nm (OD_{600 nm}) = 0.25 or until deemed cloudy visually. Previously autoclaved toothpicks were dipped in bacterial suspensions and stabbed into the sides or shoulders of bulbs. Bulbs were placed in plastic bags to maintain humidity and incubated for 4 days at 37°C or 7 days at 28°C, as deemed appropriate based on temperatures at which tested strains grew well.

Selection of genes for primer design. For *Burkholderia* spp. primers, genes were selected using the *Burkholderia* Genome Database (<http://www.burkholderia.com/>) (Winsor et al. 2008). A comparative search was performed to find genes annotated as “hypothetical protein” present in the genome sequences of strains of BCC bacteria *B. ambifaria*, *B. cenocepacia*, *B. cepacia*, *B. gladioli*, and *B. multivorans* but absent from strains of *B. mallei*, *B. pseudomallei*, and *B. thailandensis*, which do not belong to the BCC and have never been described as onion pathogens, as well as *B. vietnamiensis*, which falls within the BCC but also is reportedly not pathogenic on onion (Garrity et al. 2005). The resulting list was filtered to eliminate genes that were too short for primer design. Bamb_5978 from *B. ambifaria* and similar genes from other strains (Bcen_5856, Bcep1808_5498, Bmul_5338, and Bgla_2g16680) were chosen because they are longer than 3 kb, which would facilitate design of compatible primers.

The target gene for *P. ananatis* primers was selected from the chromosome of LMG20103 (CP001875.2) by selecting putative genes greater than 1,000 bp and annotated as encoding hypothetical proteins. These were compared against the NCBI nucleotide collection database via blastn. PANA_1080 was chosen because it was of sufficient length, and the top four matches in the blast search were *P. ananatis* (1,019 to 1,032 identities of 1,032 bp), while the fifth best match, to mouse, was poor (42 identities of 1,032 bp).

For *Enterobacter* sp. primers, chromosomal DNA sequence from *Enterobacter* strain EcWSU1 (CP002886.1), a strain originally isolated from onion, was compared with sequences from *P. ananatis*

LMG20103 (NC_013956.1) and *Klebsiella oxytoca* KCTC 1686 (CP003218.1) using progressive Mauve (Mauve version 2.3.1, build 173; Darling et al. 2010). Genes found in EcWSU1 but not the other two bacterial strains were then checked against the NCBI genomes database using blastn. EcWSU1_01990 was selected based on the absence of similarity to genes in other genera but high similarity within EcWSU1, ATCC 13047 (NC_014121.1), and NCTC 9394 (FP929040.1). These strains were all annotated as *E. cloacae* at the time of primer design.

Primer design and selection. DNA sequences for Bamb_5978, PANA_1080, and EcWSU1_01990 and similar sequences from other bacteria were collected from the NCBI genome database or the *Burkholderia* Genome Database and aligned separately using MegAlign. Candidate primer sites were chosen from well-conserved regions of the gene alignments. For *Burkholderia* spp., five forward and five reverse primers were designed based on Bamb_5978. Three forward and three reverse primers were designed based on PANA_1080. For *Enterobacter* sp., an initial set of EcWSU1_01990-based primers was found to amplify *Enterobacter* strains too generally. The sequences of amplicons from onion-field samples using this original primer set were thereafter included in additional alignments to refine the specificity of the primers. Eight forward and six reverse primers were designed based on EcWSU1_01990.

For *Burkholderia* spp. primers, all combinations of five forward and five reverse primers were tested against two strains of *B. cepacia* and one strain of *B. cenocepacia*. The primer pair that gave the highest yields of amplicons without nonspecific amplicons with the three strains (BAMB_5987 40F/350R; Table 5) was investigated further.

For *P. ananatis* primers, all combinations of three forward and three reverse primers for PANA_1080 were tested against *P. ananatis* OC5a. The primer pair that gave the highest yield of amplicons was PANA_1080 61F/1009R (Table 5). For primers intended to amplify the cluster of *Enterobacter* strains most commonly isolated from onion (*Enterobacter* sp. primers), 14 primer pair combinations were tested against EcWSU1 and two nontarget strains of *Enterobacter* as template. Primer pairs amplifying only EcWSU1 then were tested against an additional five nontarget strains of *Enterobacter*. The primer pair EcWSU1_01990 30F/443R (Table 5) was chosen for further testing because it did not yield nonspecific amplicons in the initial screens.

PCR protocols. Specific PCR primers were assessed against various strains using bacterial cells suspended in water as template. In all, 5 to 10 colonies ranging in size from 0.5 to 2.0 mm in diameter after growing on LB agar for 1 to 2 days were placed in 200 µl of sterile water using sterile wooden applicators.

Table 4. Onion-pathogenic bacteria used to assess BAMB_5978 40F/350R, PANA_1080 61F/1009R, and EcWSU1_01990 30F/443R primer pairs

Strain	Isolated from	Strain designations	Received from or reference	Amplification with		
				BAMB_5978 40F/350R	PANA_1080 61F/1009R	EcWSU1_01990 30F/443R
<i>Burkholderia cepacia</i>	Onion, 1948	ATCC 25416	Type strain of <i>B. cepacia</i>	+	–	–
<i>B. gladioli</i> pv. <i>allii</i> cola	Onion bulb rot	ATCC 19302		+	–	–
<i>Dickeya dadantii</i>		Dickey 151		–	–	–
<i>Enterobacter cloacae</i>	Onion	EcWSU1		–	–	+
<i>Erwinia rhapontici</i>	Rhubarb, England	ATCC 29283	Type strain of <i>E. rhapontici</i>	–	–	–
<i>Pantoea agglomerans</i>	Onion, South Africa.	LMG 2595; SUH 1; Eh220	M. J. Hattingh; Hattingh and Walters (1981)	–	–	–
<i>P. agglomerans</i>	Knee laceration	ATCC 27155	Type strain of <i>P. agglomerans</i>	–	–	–
<i>P. ananatis</i>	Pineapple, Brazil	ATCC 33244; LMG 2665	Type strain of <i>P. ananatis</i>	–	+	–
<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	Potato, Denmark	ATCC 15713		–	–	–
<i>Pseudomonas viridiflava</i>	Dwarf or runner bean, Switzerland	LMG 2352		–	–	–
<i>Rahnella aquatilis</i>	Drinking water, France	ATCC 33071	Type strain of <i>R. aquatilis</i>	–	–	–
<i>Xanthomonas axonopodis</i> pv. <i>allii</i>	Onion, Colorado	O274	H. Schwartz; Colorado State University	–	–	–

Reactions to test primer specificity were routinely run in 12- μ l volumes. For sensitivity assays, 25- μ l reaction volumes were used. Reaction compositions and conditions were adjusted for each primer pair accordingly (Tables 6 and 7). The lengths of annealing and elongation steps were adjusted to accommodate thermal cyclers with differing ramp rates.

Gel electrophoresis. Five microliters of each completed reaction were resolved and visualized in 1% agarose gels (Agarose LF; Amresco, Solon, OH) supplemented with SybrSafe (Life Technologies, Grand Island, NY). Gels were run in 1 $\times$ Tris-acetate-EDTA buffer and visualized on a model 3-3000 transilluminator (Fotodyne Inc., Hartland, WI). Amplicon size was estimated by comparison with a DNA ladder (2-log DNA ladder; New England Biolabs, Ipswich, MA).

Determination of primer sensitivity. Sterile cotton-tipped applicators were used to transfer *B. cepacia* strain ATCC 25416, *P. ananatis* strain OC5a, and *Enterobacter* strain EcWSU1 from LB agar plates incubated overnight into autoclaved HP water. Bacterial suspensions were prepared to an OD_{600 nm} = 0.2. Tenfold serial dilutions were prepared from each bacterial suspension, and suspensions and dilutions were used directly as templates in PCR. Additionally, suspensions and dilutions were spotted onto LB agar, and CFU per milliliter for each suspension was calculated. For our purposes, sensitivity is the order of magnitude of cells that could be amplified consistently in at least two of three repeated assays.

Determination of primer specificity. BAMB_5978 40F/350R primers were screened against 25 BCC strains that were isolated from onion previously and an additional four BCC strains isolated from soil. Additionally, the primers were tested against four *B. gladioli* strains, an additional 10 strains putatively identified as *Burkholderia* spp. but falling outside of the BCC and *B. gladioli*, and six strains from other genera, including *Pseudomonas* and *Xanthomonas* (Table 1) and additional onion-pathogenic bacteria (Table 4).

The PANA_1080 61F/1009R primer pair was vetted against more than 36 *P. ananatis* strains, 18 strains of *Pantoea* from other species (Table 2), and additional onion-pathogenic bacteria (Table 4).

The EcWSU1_01990 30F/443R primer pair was tested against 26 *E. ludwigii*-like strains isolated from onion or onion-field soil, an additional four strains of *Enterobacter* spp. from onion or onion-field environs (strains M29c, Ecl-1 GA, Ecl-2 GA, and Ecl-3 GA could not be placed unambiguously in the genus *Enterobacter*), five strains of *Enterobacter* spp. from nonagricultural sources, two strains belonging to the closely related genus *Klebsiella* (Table 3), and additional onion-pathogenic bacteria (Table 4).

Amplification of each strain was tested in three separate experiments. Failure to amplify a target strain during a repetition of the experiment occurred rarely. Failure of a target strain to amplify was typically accompanied by lack of primer dimers or any background. Primer sets were scored as having detected a strain if an amplicon

Table 5. Polymerase chain reaction primers used in this study

Primer pairs used	Primer sequence	Annealing temperature (°C)	Expected amplicon size (bp)	Source
<i>Burkholderia gladioli</i> and some BCC ^a species				
BAMB_5978 40F/350R pair		55	336	
BAMB_5978 40F	5'-GTA CGG CAA GTT CAC CGA CG-3'			This work
BAMB_5978 350R	5'-CCG AAC ATC TGC TCG AAG AA-3'			This work
BCC ^a				
BCR1/2 pair		58	1,043 ^b	
BCR1	5'-TGA CCG CCG AGA AGA GCA A-3'			Mahenthiralingam et al. 2000
BCR2	5'-CTC TTC TTC GTC CAT CGC CTC-3'			Mahenthiralingam et al. 2000
Genus <i>Burkholderia</i>				
BUR3/4 pair		57	385 ^b	
BUR3	5'-GA(AG) AAG CAG TTC GGC AA-3'			Payne et al. 2005, 2006
BUR4	5'-GAG TCG ATG ACG ATC AT-3'			Payne et al. 2005, 2006
<i>Pantoea ananatis</i>				
PANA_1080 61F/1009R pair		55	949	
PANA_1080 61F	5'-ACC CTG TCC CCG TTG GCA CTG T-3'			This work
PANA_1080 1009R	5'-AAT GAT GCC CAC TGT TGA AGG AAT-3'			This work
PanITS1/EC5 pair		51	398 ^b	
PanITS1	5'-GTC TGA TAG AAA GAT AAA GAC-3'			Gitaitis et al. 2002
EC5	5'-TGC CAG GGC ATC CAC CG-3'			Gitaitis et al. 2002
PanITS1/EC5 pair		51	398 ^b	
PanITS1	5'-GTC TGA TAG AAA GAT AAA GAC-3'			Carr et al. 2010, Gitaitis et al. 2002
AS2b	5'-TTC ATA TCA CCT TAC CGG CGC-3'			Carr et al. 2010
PanITS1/EC5 pair		60	361 or 389 ^b	
EC5 Figueiredo	5'-TGC CAG GGC ATC CAC CGT GTA CGC T-3'			Figueiredo and Paccola-Meirelles, 2012
ANAF	5'-CGT GAA ACT ACC CGT GTC TGT TGC-3'			Figueiredo and Paccola-Meirelles, 2012
<i>Enterobacter</i> sp.				
EcWSU1_01990 30F/443R pair		55	414	
EcWSU1_01990 30F	5'-GGC AAA GCT CAA CCC GGA GGT ATT CT-3'			This work
EcWSU1_01990 443R	5'-CAA AGA AAG ATA ATA ATT TCA CGG TTA GTC-3'			This work

^a BCC = *Burkholderia cepacia* complex.

^b Amplicon sizes as reported in original references.

was produced in at least two of three reactions. Strains counted as negative did not amplify in any of the three repetitions of the experiment.

Amplification of target bacteria in mixtures. Bacterial suspensions were prepared by transferring bacteria from LB agar plates into sterile HP water using sterile cotton-tipped applicators. Concentrations were adjusted to OD_{600 nm} = 0.2. For use as PCR templates, these suspensions were diluted 10-fold in sterile HP water. Mixtures of bacteria were created by mixing suspensions of target bacteria with one or two strains of nontarget bacteria, with each strain in the mixture diluted 10-fold from the suspensions at OD_{600 nm} = 0.2. Bacteria used in this assay were *B. cepacia* ATCC 25416, *Dickeya dadantii* Dickey 151, *Enterobacter* sp. EcWSU1, *P. agglomerans* SUH 1, *P. ananatis* ATCC 33244, *Pectobacterium carotovorum* ATCC 15713, and *Pseudomonas viridiflava* LMG 2352. Target bacteria were *B. cepacia* ATCC 25416 for BAMB_5978 40F/350R primers, *Pantoea ananatis* ATCC 33244 for PANA_1080 61F/1009R primers, and *Enterobacter* sp. EcWSU1 for EcWSU1_01990 30F/443R primers.

Results

Comparison of *Burkholderia* spp. and *P. ananatis* primer sets. DNA from bacterial suspensions of six strains of *Burkholderia* spp. were used as templates for the BAMB_5978 40F/350R primer pair; Bur3/4 primer pair, which produces amplicons from *Burkholderia* spp. DNA generally; and BCR1/2, which produces amplicons from DNA of members of the BCC. BAMB_5978 40F/350R amplified DNA from strains in the BCC and *B. gladioli*. Bur3/4 produced amplicons from DNA from all *Burkholderia* strains tested, and BCR1/2 produced amplicons only from DNA from the BCC strains tested, consistent with the specificities reported in the literature (Fig. 1).

Bacterial suspensions of six strains of *Pantoea* spp., one strain of *P. stewartii* subsp. *stewartii*, two of *P. agglomerans*, two of *P. allii*, and one *P. ananatis* were used as templates against the PANA_1080 61F/1009R, ANAF/EC5 (Figueiredo and Paccola-Meirelles 2012), PanITS1/EC5 (Gitaitis et al. 2002), and PanITS1/AS2b (Carr et al. 2010) primer pairs to demonstrate differences in primer specificity (Fig. 2). The PANA_1080 61F/1009R primer pair produced an amplicon only from the *P. ananatis* strain. ANAF/EC5 produced amplicons from the *P. stewartii*, *P. allii*, and *P. ananatis* strains. PanITS1/EC5 and PanITS1/AS2b produced amplicons from *P. allii* and *P. ananatis* strains.

Primer specificity. The *Burkholderia* spp.-specific primers were tested against 29 strains of *Burkholderia* from the BCC (including

25 strains from onion, 1 of which is the type strain of *B. cepacia*) and 4 strains of *B. gladioli*. These strains yielded positive reactions with the primers. An additional 10 strains identified as *Burkholderia* spp. but, falling outside of the BCC and *B. gladioli*, did not amplify with the primers. All of these strains were isolated from onion but, for nine, their *gyrB* sequences placed them closer to *B. phymatum* than to the BCC or *B. gladioli*, and these strains did not cause disease in onion in our pathogenicity tests (Supplementary Fig. S2). The primers also failed to amplify other closely related bacteria often associated with onion such as *Xanthomonas axonopodis* and several fluorescent pseudomonads (Table 1) and other non-*Burkholderia* onion pathogens tested (Table 4).

The *P. ananatis* primers amplified 36 strains of *P. ananatis*, including 21 strains isolated from onion grown in New York and several other strains that originated elsewhere. The primers failed to amplify strains of *P. allii*, a closely related species reported to cause disease in onion. The primers also failed to amplify the *P. agglomerans* strains tested, which included both pathogenic and nonpathogenic strains (Table 2).

The *Enterobacter* sp. primers produced an amplicon of the appropriate size for all 26 strains that clustered with EcWSU1 and *E. ludwigii*, based on their *gyrB* sequences. The primers failed to amplify DNA from all other strains tested, including two *Enterobacter* spp. strains and four strains closely related to *Enterobacter*, all isolated from onion; two *Enterobacter* spp. strains isolated from onion-field soil or water; five *Enterobacter* strains from other sources; and two strains of the closely related genus *Klebsiella* (Table 3).

Additionally, BAMB_5978 40F/350R, PANA_1080 61F/1009R, and EcWSU1_01990 30F/443R were tested against 13 strains of species that are pathogenic to onion or are frequently found associated with onion from nine genera (Table 4). Primers were found to specifically amplify DNA from target bacteria when other bacteria were present that might also be found in diseased onion bulbs (Fig. 3).

Primer sensitivity. For each primer set (BCC- and *B. gladioli*-specific primers BAMB_5978 40F/350R, *P. ananatis*-specific primers PANA_1080 61F/1009R, and *Enterobacter* sp. primers EcWSU1_01990 30F/443R), the minimum number of CFU detectable per 25- μ l reaction mixture was approximately 10³. The OneTaq Standard Reaction Buffer and OneTaq GC Reaction Buffer were tested with the EcWSU1_01990 30F/443R primer pair to compare sensitivity and specificity. Primer specificity was similar with both buffers but sensitivity assays with bacterial suspensions as templates were more consistent and, on average, more sensitive using the GC Reaction Buffer.

Use of primers with mixed bacterial cultures. BAMB_5978 40F/350R, PANA_1080 61F/1009R, and EcWSU1_01990 30F/443R primer pairs were tested against pure cultures of a single bacterial strain and against mixtures containing an appropriate target strain and an additional one or two strains of nontarget bacteria. In each case, primer sets detected the target strains singly or in the presence of one or two other bacteria (Fig. 4).

Discussion

To design specific primers, we used genome sequences available in GenBank and other public databases to identify genes present in the bacteria we wished to detect but generally absent from nontarget

Table 6. Polymerase chain reaction compositions used in this study^a

Reagents used	Final concentration
5x OneTaq GC Reaction Buffer	1x
dNTPs	0.25 mM
Primer 1	0.52 μ M
Primer 2	0.52 μ M
OneTaq	0.3 units/12 μ l reaction
Template	2 μ l
Water	To volume

^a Standard composition was used for all reactions. Reaction volumes were 12 or 25 μ l.

Table 7. Cycling conditions used in this study^a

Conditions ^b	EcWSU1_01990 30F/443R	BAMB_5978 40F/350 R	PANA_1080 61F/1009R	BCR1/2	BUR3/4	ANAF/EC5 Figueiredo	Pan ITS1/EC5 and PanITS1/AS2b
Annealing (°C)	55	55	55	58	57	60	51
Extension	45 s to 1 min 10 s	40 s	45 s to 1 min 15 s	70 s	45 s	30 to 45 s	30 to 45 s
Steps repeated	45	45	45	30	30	30	30

^a Parameters are listed for each primer pair. Duration of steps 3 and steps 4 was adjusted to accommodate thermal cyclers with differing ramp speeds.

^b Generic cycling parameters were standard, with three variable parameters: annealing temperatures, extension time, and the number of times steps 2 through 4 were repeated in total. Step 1: 95°C for 10 min, step 2: 95°C for 30 s, step 3: annealing temperature for 30 to 45 s, step 4: extension time at 72°C, step 5: 72°C for 10 min, and step 6: hold at 12°C.

bacteria. This strategy was intended to make false positives unlikely because nontarget bacteria would completely lack the gene containing the primer pair's annealing sites. For each primer pair designed, we paid little attention to the putative function of the gene on which the sequence of the primer pairs were based. However, without a clear understanding of the target gene's role or the phenotypic consequences of its mutation or loss, it is possible to choose a priming site that might be altered or deleted with little fitness cost. Under such circumstances, one might find populations of the target organism that are not amplifiable, yielding false negative results. To reduce that possibility, we did extensive empirical testing of the new primer sets with many strains. For the three groups of target bacteria we addressed, the novel primer pairs designed proved highly effective and specific for their respective target bacteria.

For the genus *Burkholderia*, particularly members of the BCC, species continue to be delineated and the genus has changed substantially over the last two decades (Vandamme and Peeters 2014). Numerous primer sets for detecting various bacteria of the genus *Burkholderia* exist but the intended specificities of these primers differ from the specificity of the BAMB_5978 primer pair we report here. BAMB_5978 primers were designed to amplify *B. gladioli* and those BCC species likely to be pathogenic to onion. The candidate primer pairs were vetted against numerous *Burkholderia* strains isolated from onion. The *Burkholderia* spp.-specific primers amplified all strains isolated from onion that were identified as belonging to the BCC or *B. gladioli*. They failed to amplify nine nontarget isolates from onion bulbs identified as *Burkholderia* spp. based on their *gyrB* sequence. The sequence of *gyrB* placed these strains as more

Fig. 1. Primer pair BAMB_5978 amplifies representatives of the *Burkholderia cepacia* complex and *B. gladioli*. Three different primer pairs were tested using bacterial suspensions of *B. cepacia*, *B. gladioli*, other *Burkholderia* spp., or water as template. **A**, BAMB_5978 40F/350R primer pair; **B**, BUR3/4 primer pair (Payne et al. 2005); and **C**, BCR1/2 primer pair (Mahenthalingam et al. 2000). Lane 1, *B. cepacia* ATCC 25416 (type strain); lane 2, *B. cepacia* ATCC 39277; lane 3, *B. gladioli* ATCC 19302; lane 4, *B. gladioli* PA-23; lane 5, *Burkholderia* sp. H49a; lane 6, *Burkholderia* sp. O64a; and lane 7, water. Lanes designated "L" were loaded with 2-log DNA ladder (New England Biolabs).

Fig. 2. Primer pair PANA_1080 61F/1009R amplifies *Pantoea ananatis* specifically. Four different primer pairs were tested using bacterial suspensions of three different *Pantoea* spp. or water as template. **A**, PANA_1080 61F/1009R primer pair; **B**, ANAF/EC5 (Figueiredo and Paccola-Meirelles 2012); **C**, PanITS1/EC5 (Gitaitis et al. 2002); and **D**, PanITS1/AS2b (Carr et al. 2010). Lane 1, *P. stewartii* subsp. *stewartii* DC283; lane 2, *P. agglomerans* ATCC 27155; lane 3, *P. agglomerans* SUH1; lane 4, *P. allii* LMG 24202; lane 5, *P. allii* LMG 24248; lane 6, *P. ananatis* Pna97-1; and lane 7, water. Lanes designated "L" were loaded with 2-log DNA ladder (New England Biolabs).

closely related to *B. phymatum* and *B. bryophila* than to BCC strains or *B. gladioli*. Work by other groups using the 16S ribosomal DNA sequence and multilocus sequence analysis (MLSA) demonstrated that *B. phymatum* and *B. bryophila* reside in a distinct clade within the genus *Burkholderia* that is separate from the BCC and *B. gladioli* (Estrada-de los Santos et al. 2013). We are unaware of any reports that strains from this clade cause disease in onion. In our pathogenicity tests in onion bulbs, the tested strains did not cause symptoms.

Onion plants are susceptible to diseases caused by *P. ananatis* and its close relatives *P. allii* and *P. agglomerans*, pathogenic strains of which all cause similar symptoms. The PANA_1080 61F/1009R primer pair was tested against numerous strains of the genus *Pantoea*, including many *P. ananatis* and *P. agglomerans* strains isolated from onion. The primers consistently amplified strains of *P. ananatis* but not other species of *Pantoea*, suggesting that the primer pair is sufficiently specific to selectively detect *P. ananatis*. To demonstrate the differing specificities of PANA_1080 61F/1009R and previously published primers for detection of *P. ananatis*, several primer sets were tested against six strains of *Pantoea*, including one *P. stewartii*, two *P. agglomerans*, two *P. allii*, and one *P. ananatis* strain. In our hands, the PanITS1/EC5, PanITS1/AS2b, and ANAF/EC5 primer pairs all produced amplicons from the *P. ananatis* strain and two *P. allii* strains tested. Additionally, ANAF/EC5 but not PanITS1/EC5 or PanITS1/AS2b produced an amplicon with *P. stewartii* subsp. *stewartii* DC283. The amplification of *P. allii* strains was consistent with the specificity of ANAF/EC5 reported previously (Figueiredo and Paccola-Meirelles 2012) and was not reported previously for PanITS1/EC5 (Gitaitis et al. 2002) or PanITS1/AS2b (Carr et al. 2010) because the primers were described prior to the publication of *P. allii* as a species (Brady et al. 2011). Detection of *P. ananatis* and *P. allii* together may be desirable in some cases but, in cases where it is not, PANA_1080 61F/1009R is more discriminatory. The amplification of *P. stewartii* subsp. *stewartii* DC283 by the ANAF/EC5 primer pair but not PanITS1/EC5 or PanITS1/AS2b was unexpected, because amplification of *P. stewartii* strains was previously noted for PanITS1/EC5 (Gitaitis et al. 2002) but not for ANAF/EC5 (Figueiredo and Paccola-Meirelles 2012). This discrepancy may be due to the fact that the strain of *P. stewartii* used in our assay differed from those used in the original

publications. None of the primer sets assessed produced amplicons from *P. agglomerans* strains, which is consistent with published data.

The state of the genus *Enterobacter* initially proved to be problematic for primer design. The genus was not monophyletic and the use of common biochemical tests for identification of strains often led to misidentification (Kämpfer et al. 2008). It became clear that it was not possible to rely on the accurate identification of genome-sequenced strains for a genus in which common identification methods were unreliable and relationships among species unclear. Based on recent MLSA of many strains that represent several species of *Enterobacter*, Brady et al. (2013) proposed changes to the genus. How the recommended changes and the availability of MLSA data for numerous strains will affect the taxonomic position of the sequenced *Enterobacter* strains in GenBank is in question. Because the identification of *Enterobacter* strains in GenBank is not always reliable, we relied on a sequence that we obtained directly from *Enterobacter* strains isolated from onion to inform our decisions about which strains would be most useful for detection. We found that most strains isolated from onion had very similar partial *gyrB* sequences, and we used this sequence to identify strains with publicly available genome sequence that were likely closely related to our strains of interest. When tested against numerous *Enterobacter* strains, the EcWSU1_01990 primer set amplified only *Enterobacter* strains that clustered together based on *gyrB* sequence.

The new primer pairs have been tested against numerous bacterial strains and appear to be highly specific for their intended target bacteria. However, the primers are not exceedingly sensitive, requiring 10^3 CFU per 25 μ l of reaction mixture. In protocols including a culturing step in order to separate bacteria from sample substrates containing PCR inhibitors, such as onion bulb tissue or high-organic matter muck-land soil, typically used for growth of onion in New York, the required number of bacteria are easily obtained. However, the low sensitivity of our primer assays may limit the utility of these primers in protocols lacking a culturing step.

The primers reported herein have proven useful for detection of the three groups of onion pathogens common in New York State. However, regardless of the geographic region from which they were

Fig. 3. Primer pairs for specifically detecting *Burkholderia* strains, including onion pathogens, *Pantoea ananatis*, or the group of *Enterobacter* strains most commonly isolated from onion that amplify their target bacteria specifically. Primer pairs were tested against bacterial suspensions or water as templates. **A**, BAMB_5978 40F/350R primer pair; **B**, PANA_1080 61F/1009R primer pair; and **C**, EcWSU1_01990 30F/443R primer pair. Lane 1, *Burkholderia cepacia* ATCC 25416 (type strain); lane 2, *B. gladioli* pv. *allii* ATCC 19302; lane 3, *Dickeya dadantii* Dickey 151; lane 4, *Enterobacter* sp. EcWSU1; lane 5, *Erwinia rhapontici* ATCC 29283; lane 6, *P. agglomerans* SUH1; lane 7, *P. agglomerans* ATCC 27155; lane 8, *P. ananatis* ATCC 33244; lane 9, *Pectobacterium carotovorum* ATCC 15713; lane 10, *Pseudomonas viridiflava* LMG 2352; lane 11, *Rahnella aquatilis* ATCC 33071; lane 12, *Xanthomonas axonopodis* pv. *allii* O274; and lane 13, water. Lanes designated "L" were loaded with 2-log DNA ladder (New England Biolabs).

Fig. 4. Primer pairs **A**, PANA_1080 61F/1009R; **B**, EcWSU1_01990 30F/443R; and **C**, BAMB_5978 40F/350R amplify from target bacteria using pure or mixed bacterial suspensions as templates. Polymerase chain reactions (PCR) or ladder (5 μ l) were loaded into each well. PCR are designated by the bacterial strains used as templates. L = 2-log ladder (New England Biolabs), Bc = *Burkholderia cepacia* ATCC 25416, Dd = *Dickeya dadantii* Dickey 151, Ec = *Enterobacter cloacae* EcWSU1, Pag = *Pantoea agglomerans* ATCC 27155, Pan = *P. ananatis* ATCC 33244, Pc = *Pectobacterium carotovorum* ATCC 15713, and Pv = *Pseudomonas viridiflava* LMG 2352.

isolated, target strains included in assays of primer specificity were successfully amplified. Our primer pairs, which target single-copy genes and produce discrete, qualitative results, have been assessed specifically for use in detecting pathogens of onion. Thus far, they have proven to be highly specific, each giving few false-negative and no false-positive results. Because primer sets are specific, the primers can be used for mixed cultures or environmental samples, assuming that the bacterial titers are sufficient and inhibition of the PCR is not a problem.

The design and validation of these three primer sets for detection of species of onion-pathogenic bacteria should prove useful for labs investigating bacterial decays of onion bulbs. We leveraged the availability of numerous bacterial genomes in public databases to identify genes present in certain bacteria but absent from the genomes of non-target bacteria that occupy the same or similar niches. For this primer design strategy to succeed, numerous sequenced genomes of both target bacteria and taxonomically or ecologically related bacteria were required for analysis. Such an approach would not have been possible even 10 years ago. The strategy may have utility for design of primers for other target bacteria of clinical, agricultural, or environmental significance.

Acknowledgments

Funding in support of this study was provided to S. V. Beer by New York State Specialty Crops Block Grants; a Northeast Regional IPM grant to B. Gugino (Pennsylvania State University) and C. A. Hoepting (Cornell Cooperative Extension, Albion, NY); the United States Department of Agriculture Multi-State Regional Hatch Project W-2008 for research on *Iris yellow spot virus*, thrips, and other diseases of onion; the New York State Onion Research and Development Program; and Cornell University. We thank onion growers in New York State for gifts of symptomatic onion bulbs from which many pathogens included in these studies were isolated; R. Gitaitis, B. Schroeder, and H. Schwartz for the kind gifts of strains used in panels to determine primer specificity; and B. Schroeder for disclosing unpublished results.

Literature Cited

- Bishop, A. L., and Davis, R. M. 1990. Internal decay of onions caused by *Enterobacter cloacae*. *Plant Dis.* 74:692-694.
- Bonasera, J. M., Asselin, J. E., and Beer, S. V. 2014. Identification of bacteria pathogenic to or associated with onion (*Allium cepa*) based on sequence differences in a portion of the conserved gyrase B gene. *J. Microbiol. Methods* 103:138-143.
- Brady, C., Cleenwerck, I., Venter, S., Coutinho, T., and De Vos, P. 2013. Taxonomic evaluation of the genus *Enterobacter* based on multilocus sequence analysis (MLSA): Proposal to reclassify *E. nimipressuralis* and *E. amnigenus* into *Lelliottia* gen. nov. as *Lelliottia nimipressuralis* comb. nov. and *Lelliottia amnigena* comb. nov., respectively, *E. gergoviae* and *E. pyrinus* into *Pluralibacter* gen. nov. as *Pluralibacter gergoviae* comb. nov. and *Pluralibacter pyrinus* comb. nov., respectively, *E. cowanii*, *E. radicincitans*, *E. oryzae* and *E. arachidis* into *Kosakonia* gen. nov. as *Kosakonia cowanii* comb. nov., *Kosakonia radicincitans* comb. nov., *Kosakonia oryzae* comb. nov. and *Kosakonia arachidis* comb. nov., respectively, and *E. turicensis*, *E. helveticus* and *E. pulveris* into *Cronobacter* as *Cronobacter zurichensis* nom. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov., respectively, and emended description of the genera *Enterobacter* and *Cronobacter*. *Syst. Appl. Microbiol.* 36: 309-319.
- Brady, C., Cleenwerck, I., Venter, S., Vancanneyt, M., Swings, J., and Coutinho, T. 2008. Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). *Syst. Appl. Microbiol.* 31:447-460.
- Brady, C. L., Goszczynska, T., Venter, S. N., Cleenwerck, I., Vos, P. d., Gitaitis, R. D., Coutinho, T. A., and de Vos, P. 2011. *Pantoea allii* sp. nov., isolated from onion plants and seed. *Int. J. Syst. Evol. Microbiol.* 61:932-937.
- Burkholder, W. H. 1942. Three bacterial plant pathogens: *Phytomonas caryophylli* sp. n., *Phytomonas alliicola* sp. n., and *Phytomonas manihotis* (Arthaud-Berthet et Bondar) Viegas. *Phytopathology* 32:141-149.
- Burkholder, W. H. 1950. Sour skin, a bacterial rot of onion bulbs. *Phytopathology* 40:115-117.
- Carr, E. A., Bonasera, J. M., Zaid, A. M., Lorbeer, J. W., and Beer, S. V. 2010. First report of bulb disease of onion caused by *Pantoea ananatis* in New York. *Plant Dis.* 94:916.
- Carr, E. A., Zaid, A. M., Bonasera, J. M., Lorbeer, J. W., and Beer, S. V. 2013. Infection of onion leaves by *Pantoea ananatis* leads to bulb infection. *Plant Dis.* 97:1524-1528.
- Darling, A. E., Mau, B., and Perna, N. T. 2010. Progressive Mauve: Multiple genome alignment with gene gain, loss and rearrangement. *PLoS One* 5: e11147.

- De Smet, B., Mayo, M., Peeters, C., Zlosnik, J. E. A., Spilker, T., Hird, T. J., LiPuma, J. J., Kidd, T. J., Kaestli, M., Ginther, J. L., Wagner, D. M., Keim, P., Bell, S. C., Jacobs, J. A., Currie, B. J., and Vandamme, P. 2015. *Burkholderia stagnalis* sp. nov. and *Burkholderia territorii* sp. nov., two novel *Burkholderia cepacia* complex species from environmental and human sources. *Int. J. Syst. Evol. Microbiol.* 65:2265-2271.
- Dolph, P. J., Majerczak, D. R., and Coplin, D. L. 1988. Characterization of a gene cluster for exopolysaccharide biosynthesis and virulence in *Erwinia stewartii*. *J. Bacteriol.* 170:865-871.
- Estrada-de los Santos, P., Vinuesa, P., Martínez-Aguilar, L., Hirsch, A. M., and Caballero-Mellado, J. 2013. Phylogenetic analysis of *Burkholderia* species by multilocus sequence analysis. *Curr. Microbiol.* 67:51-60.
- Figueiredo, J. E. F., and Paccola-Meirelles, L. D. 2012. Short communication: Simple, rapid and accurate PCR-based detection of *Pantoea ananatis* in maize, sorghum and *Digitaria* sp. *J. Plant Pathol.* 94:663-667.
- Garity, G. M., Bell, J. A., and Lilburn, T. 2005. Class II. Betaproteobacteria class. nov. Pages 575-922 in: Bergey's Manual of Systematic Bacteriology, Vol. 2, part C, The Proteobacteria. Brenner, D. J., Krieg, N. R., and Staley, J. T., eds. Springer, New York.
- Gitaitis, R., Walcott, R., Culpepper, S., Sanders, H., Zolobowska, L., and Langston, D. 2002. Recovery of *Pantoea ananatis*, causal agent of center rot of onion, from weeds and crops in Georgia, USA. *Crop Prot.* 21:983-989.
- Goszczynska, T., Moloto, V. M., Venter, S. N., and Coutinho, T. A. 2006a. Isolation and identification of *Pantoea ananatis* from onion seed in South Africa. *Seed Science Technol.* 34:655-668.
- Goszczynska, T., Venter, S. N., and Coutinho, T. A. 2006b. PA 20, a semi-selective medium for isolation and enumeration of *Pantoea ananatis*. *J. Microbiol. Methods* 64:225-231.
- Hattingh, M. J., and Walters, D. F. 1981. Stalk and leaf necrosis of onion caused by *Erwinia herbicola*. *Plant Dis.* 65:615-618.
- Humann, J. L., Wildung, M., Cheng, C. H., Lee, T., Stewart, J. E., Drew, J. C., Triplett, E. W., Main, D., and Schroeder, B. K. 2011. Complete genome of the onion pathogen *Enterobacter cloacae* EcWSU1. *Stand. Genomic Sci.* 5:279-286.
- Kämpfer, P., Nienhüser, A., Packroff, G., Wernicke, F., Mehling, A., Nixdorf, K., Fiedler, S., Kolauch, C., and Esser, M. 2008. Molecular identification of coliform bacteria isolated from drinking water reservoirs with traditional methods and the Colilert-18 system. *Int. J. Hyg. Environ. Health* 211:374-384.
- Kido, K., Hasegawa, M., Matsumoto, H., Kobayashi, M., and Takikawa, Y. 2010. *Pantoea ananatis* strains are differentiated into three groups based on reactions of tobacco and Welsh onion and on genetic characteristics. *J. Gen. Plant Pathol.* 76:208-218.
- Li, G., Hu, Z., Zeng, P., Bing, Z., and Wu, L. 2015. Whole genome sequence of *Enterobacter ludwigii* type strain EN-119^T, isolated from clinical specimens. *FEMS Microbiol. Lett.* 362:fnv033.
- Mahenthiralingam, E., Bischof, J., Byrne, S. K., Radomski, C., Davies, J. E., Av-Gay, Y., and Vandamme, P. 2000. DNA-Based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* genomovars I and III. *J. Clin. Microbiol.* 38:3165-3173.
- Payne, G. W., Ramette, A., Rose, H. L., Weightman, A. J., Jones, T. H., Tiedje, J. M., and Mahenthiralingam, E. 2006. Application of a *recA* gene-based identification approach to the maize rhizosphere reveals novel diversity in *Burkholderia* species. *FEMS Microbiol. Lett.* 259:126-132.
- Payne, G. W., Vandamme, P., Morgan, S. H., LiPuma, J. J., Coenye, T., Weightman, A. J., Jones, T. H., and Mahenthiralingam, E. 2005. Development of a *recA* gene-based identification approach for the entire *Burkholderia* genus. *Appl. Environ. Microbiol.* 71:3917-3927.
- Peeters, C., Zlosnik, J. E., Spilker, T., Hird, T. J., LiPuma, J. J., and Vandamme, P. 2013. *Burkholderia pseudomultivorans* sp. nov., a novel *Burkholderia cepacia* complex species from human respiratory samples and the rhizosphere. *Syst. Appl. Microbiol.* 36:483-489.
- Schroeder, B. K., and du Toit, L. J. 2009. First report of *Enterobacter cloacae* causing onion bulb rot in the Columbia Basin of Washington State. *Plant Dis.* 93:323.
- Schroeder, B. K., Waters, T. D., and du Toit, L. J. 2010. Evaluation of onion cultivars for resistance to *Enterobacter cloacae* in storage. *Plant Dis.* 94:236-243.
- Schwartz, H. F., and Mohan, S. K. 2008. Compendium of Onion and Garlic Diseases and Pests, 2nd ed. American Phytopathological Society, St. Paul, MN.
- Schwartz, H. F., and Otto, K. 2000. First report of a bulb decay of onion by *Enterobacter cloacae* in Colorado. *Plant Dis.* 84:808.
- Sotokawa, N., and Takikawa, Y. 2004. Occurrence of bacterial rot of onion bulbs caused by *Burkholderia cepacia* in Japan. *J. Gen. Plant Pathol.* 70:348-352.
- Vandamme, P., and Peeters, C. 2014. Time to revisit polyphasic taxonomy. *Antonie Leeuwenhoek* 106:57-65.
- Whitby, P. W., Pope, L. C., Carter, K. B., LiPuma, J. J., and Stull, T. L. 2000. Species-specific PCR as a tool for the identification of *Burkholderia gladioli*. *J. Clin. Microbiol.* 38:282-285.
- Winsor, G. L., Khaira, B., Van Rossum, T., Lo, R., Whiteside, M. D., and Brinkman, F. S. L. 2008. The *Burkholderia* Genome Database: Facilitating flexible queries and comparative analyses. *Bioinformatics* 24:2803-2804.
- Yohalem, D. S., and Lorbeer, J. W. 1994. Intraspecific metabolic diversity among strains of *Burkholderia cepacia* isolated from decayed onions, soils, and the clinical environment. *Antonie Leeuwenhoek* 65:111-131.
- Zaid, A. M., Bonasera, J. M., and Beer, S. V. 2011. First report of *Enterobacter* bulb decay of onions caused by *Enterobacter cloacae* in New York. *Plant Dis.* 95:1581.
- Zaid, A. M., Bonasera, J. M., and Beer, S. V. 2012. OEM—A new medium for rapid isolation of onion-pathogenic and onion-associated bacteria. *J. Microbiol. Methods* 91:520-526.