A Real-Time PCR Differentiating *Pantoea stewartii* subsp. *stewartii* From *P. stewartii* subsp. *indologenes* in Corn Seed

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Abstract

Stewart's wilt of corn caused by the bacterium *Pantoea stewartii* subsp. *stewartii* is a seed-borne disease of major phytosanitary importance. Many countries have imposed restrictions on corn seed imports from regions where the disease occurs to prevent the potential introduction of the pathogen. Current laboratory testing methods (enzyme-linked immunosorbent assay [ELISA] and polymerase chain reaction [PCR]) cannot readily distinguish *P. stewartii* subsp. *stewartii* from the closely related subspecies *Pantoea stewartii* subsp. *indologenes*. However, *P. stewartii* subsp. *indologenes*, a nonpathogen on corn, is occasionally found on corn seed as part of the resident bacterial population and can yield false positive test results. A real-time PCR targeting the *cpsAB* intergenic

sequence was developed to specifically detect *P. stewartii* subsp. *stewartii* from corn seeds and distinguish it from *P. stewartii* subsp. *indologenes*. The assay successfully detected *P. stewartii* subsp. *stewartii* from corn seed, and *P. stewartii* subsp. *indologenes*-contaminated seed lots, which previously yielded false positives by ELISA and published PCR methods, were negative. The absence of *P. stewartii* subsp. *stewartii* and the presence of *P. stewartii* subsp. *indologenes* in this seed were confirmed by size differentiation of the *cpsAB* amplicons in a conventional PCR. By distinguishing the two subspecies, the assays described would avoid false positive results and help prevent unnecessary restrictions on international movement of corn seed.

Pantoea stewartii subsp. *stewartii* (Smith 1898) Mergaert et al. 1993 (syn. *Erwinia stewartii* [Smith 1898] Dye 1963) (Dye 1963; Mergaert et al. 1993) is the causal agent of Stewart's wilt, also called Stewart's disease or Stewart's bacterial wilt of corn. The main hosts for the bacterium include flint corn, sweet corn, yellow dent (field) corn, popcorn, teosinte, and eastern gamagrass. There are two phases describing symptoms of Stewart's wilt: the wilt phase and the leaf blight phase. The wilt phase occurs when highly susceptible plants become systemically infected during early vegetative growth. The host plant's xylem tissue is colonized by bacterial cells, which produce an extracellular polysaccharide (EPS) called stewartan that plugs the xylem vessels and causes wilting. Infected seedlings may die prematurely. Plants that are not killed may remain stunted and produce bleached dead tassels. Dent (field) corn is generally less susceptible than sweet corn to the wilt phase, and although some inbred lines are susceptible, dent corn hybrids are usually resistant. Significant losses in yield from susceptible and moderately susceptible sweet corn hybrids have been reported (Suparyono and Pataky 1989). The leaf blight phase of the disease is more common but less damaging than the wilt phase. It can occur at any growth stage and is generally associated with local infection of the leaf tissue via feeding by the corn flea beetle vector *Chaetocnema pulicaria* Melsheimer. Symptoms develop as gray-green to yellow-green streaks with wavy margins along the veins,

and they are most noticeable after tasseling. The streaked areas eventually turn necrotic and straw colored. If the disease is severe, entire leaves may die prematurely, and it also predisposes the plant to fungal stalk rots (Jardine and Claflin 2016).

The corn flea beetle is the primary vector of the bacterium (Elliott and Poos 1934, 1940). Adult beetles harbor the bacteria in their gut, emerge in the spring about the time that corn is planted, and disseminate the bacteria throughout the corn-growing season. The bacterium enters the plant through feeding wounds and colonizes intercellular spaces of leaf tissue as well as xylem, thereby causing characteristic water-soaked lesions and wilting. Plant-to-plant spread does not occur without the presence of corn flea beetle feeding, and disease incidence is directly related to the size of flea beetle population (Cook et al. 2005). Some grassy weeds have been shown to be symptomless carriers of the bacterium, but their role as overwintering hosts is not well known (Jardine and Claflin 2016).

P. stewartii subsp. *stewartii* is indigenous to North America, having first been reported on Long Island in New York State in 1897 (Stewart 1897). The disease has occasionally been reported in other parts of the world (Canada, Mexico, Austria, Argentina, Bolivia, Italy, Poland, Romania, Russia, China, Korea, Malaysia, Thailand, and Vietnam) (CABI 2018), but the pathogen is not well established in these areas, probably because of the absence of a suitable insect vector. The economic importance of the disease in North America has declined since the 1930s with the introduction of more resistant hybrids and the common use of systemic seed-applied insecticides. Seed transmission is rare, and the likelihood of seed transmission is low from commercial seed lots; however, the risk is still more than zero if infected seeds are present. Block et al. (1998) reported a seed transmission rate of 0.022% from naturally infected seeds (one infected plant from 4,563 infected kernels), and Michener et al. (2002) reported a similar seed transmission rate of 0.038% (22 infected plants from 58,300 infected kernels). However, Stewart's wilt is still a major issue when seed is destined for export; >100 countries impose phytosanitary restrictions on importation of corn seed and require phytosanitary certification that the shipment is free of *P. stewartii*. These phytosanitary restrictions have a large impact on the corn seed industry and seed trade in terms of seed health testing costs and potential loss of export markets. Hence, quick and accurate detection of *P. stewartii* subsp. *stewartii* is very important.

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Many testing methods have been developed for the detection of *P. stewartii* in pure cultures, infected corn leaf tissue, or seeds (Blakemore et al. 1999; Coplin et al. 2002; Gehring et al. 2014; Lamka et al. 1991; Nechwatal et al. 2018; Tambong 2015; Tambong et al. 2008; Thapa et al. 2012; Uematsu et al. 2015; Wensing et al. 2010; Wilson et al. 1994; Wu et al. 2007; Xu et al. 2010). Enzyme-linked immunosorbent assay (ELISA), a serological method (Lamka et al. 1991) based on polyclonal capture and monoclonal detection antibodies, is widely used in the United States for commercial seed certification. Block et al. (2011) found that ELISA and nine published polymerase chain reaction (PCR) methods were prone to occasional false positive cross-reactions with, at the time, unidentified *Pantoea* isolates from tropical or subtropical corn seed. The unknowns were later identified by multilocus sequencing as *Pantoea stewartii* subsp. *indologenes* Mergaert et al. 1993, first described by Mergaert et al. (1993). *P. stewartii* subsp. *indologenes* can cause leaf spots on foxtail millet (*Setaria italica*) and pearl millet (*Pennisetum americanum*), rot of pineapple (*Ananas comosus*), and center rot of onion (Stumpf et al. 2018); however, it is avirulent on corn, and it is not a regulated corn pest in any country. *P. stewartii* subsp. *indologenes* is occasionally present on corn seed of tropical or subtropical origin as part of resident bacterial population (Block et al. 2011; Gehring et al. 2014; Nechwatal et al. 2018; Tambong 2015).

Thapa et al. (2012) developed a multiplex PCR for *P. stewartii* subsp. *stewartii* with three primer pairs targeting three different genes, *wtsE* (water-soaking), *cpsA* (EPS production) and *hrpN* genes (hypersensitive response in nonhost), and each primer pair was reported as being specific to *P. stewartii* subsp. *stewartii*. Nechwatal et al. (2018), in a PCR validation study, showed evidence of cross-reactivity with *P. stewartii* subsp. *indologenes* using the primers of Thapa et al. (2012) and also stated that the recommended EPPO Diagnostic Protocol PM 7/60 (EPPO 2016) was prone to false positives. Nechwatal et al. (2018) evaluated several primer sets and concluded that the *galE* primers of Gehring et al. (2014) were capable of differentiating the two subspecies based on single-nucleotide polymorphisms (SNPs). The SNPs are present as the last base at the 3' end of the forward and reverse primers. The *galE* primers were validated with three *P. stewartii* subsp. *indologenes* isolates from corn and seven isolates from other plant hosts plus a small number of corn seed samples (Gehring et al. 2014; Nechwatal et al. 2018). Although promising, the SNP primers would benefit from testing additional *P. stewartii* subsp. *indologenes* isolates, especially from corn. Little is known about variability in *P. stewartii* subsp. *indologenes* isolates and the risks associated with reliance on single-base pair differences for identification.

The objectives of this study were to (1) develop a convenient, reliable real-time PCR assay for specific detection of *P. stewartii* subsp. *stewartii* from corn seeds without the need for bacterial isolation, biochemical characterization of isolates (e.g., indole and esculin), and additional PCR tests to confirm bacterial identity and (2) clearly distinguish *P. stewartii* subsp. *stewartii* from *P. stewartii* subsp. *indologenes*.

Materials and Methods

Bacterial strains. All bacterial strains (Table 1) were cultured on Luria–Bertani (tryptone, 1%; yeast extract, 0.5%; NaCl, 1%; and agar, 1.5%) or nutrient broth yeast extract agar (nutrient broth, 0.8%; yeast extract, 0.2%; K₂HPO₄, 0.2%; KH₂PO₄, 0.05%; dextrose, 0.5%; MgSO₄·7H₂O, 0.025%; and agar, 1.5%) at 28°C for 1 to 3 days. Sixty-seven *Pantoea* isolates that included 26 *P. stewartii* subsp. *stewartii*, 17 *P. stewartii* subsp. *indologenes*, 11 *Pantoea agglomerans*, and 13 *Pantoea ananatis* from multiple hosts plus 7 non-*Pantoea* isolates were tested by ELISA and six conventional and three real-time PCR methods, and they were characterized by various physiological, biochemical, and pathogenicity tests (Table 1). Physiological and biochemical characteristics included gram staining (KOH test), motility test, Hugh–Leifson oxidation/fermentation test, indole production, and esculin hydrolysis. Genomic DNA was extracted by using the Wizard Genomic DNA

purification kit (Promega) and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Corn seeds of the accessions (entries with Ames, NSL, or PI numbers) listed in Tables 2 and 3 were acquired from the U.S. National Plant Germplasm System collection in Ames, Iowa.

ELISA. ELISA tests of bacterial cultures and corn seeds were performed according to the manufacturer's instructions for the *P. stewartii* reagent set (Agdia Inc.).

Pathogenicity test on corn seedlings. Ten-day-old corn inbred Mt42 seedlings were inoculated by stem injection with the respective bacterial cultures (Table 1) under greenhouse conditions (28°C day/24°C night). One milliliter of inoculum (~10⁸ CFU/ml) in 1× phosphate buffered saline (PBS), pH 7.4, containing 0.02% Tween-20 was injected into stems near the base of leaf whorl with a 26-gauge tuberculin syringe, and seedlings were evaluated for symptoms 8 to 10 days after inoculation. Control seedlings were inoculated with buffer alone.

Comparison of bacterial extraction procedures from seed. Two buffers, 1× PBS, pH 7.4, and 0.85% normal saline with 0.02% Tween-20, were compared with sterile milliQ water for their efficiency in extracting the target (*P. stewartii* subsp. *stewartii*) bacteria from corn seeds (Table 4). Three seed lots were tested: (1) LN1 with 3.5% infected seeds (seed infection percentage determined by single-seed ELISA), (2) AN1 with 9% infected seeds (both from naturally infected inbred A632Ht), and (3) AR4 with 37% infected seeds harvested from an inoculated corn inbred A632Ht (Block et al. 1998). Fifty whole corn seeds from each of the three lots were incubated in 50 ml of 1× PBS buffer, 50 ml of saline solution, or 50 ml of milliQ water at room temperature on a shaker at 160 rpm for 4 h. Seed washes were processed as described below in the section on SYBR Green real-time PCR. The experiment was repeated twice with three replicate samples per run.

To compare the sensitivity of detection using whole seed versus ground seed, ground seed mixtures from 50 seeds per lot were incubated with either sterile PBS or sterile water at room temperature for 30 min with occasional mixing. A 1.2-ml aliquot was centrifuged at 1,000 relative centrifugal force (RCF) for 5 min to pellet corn debris, and the supernatant was transferred to a new tube; 2 µl of the supernatant was used directly for real-time PCR after an initial denaturation at 95°C for 10 min.

Primer design. Primers were designed from the intergenic DNA sequence present between the *cpsA* and *cpsB* genes of the *wce-I* operon. The *wce-I* operon consists of 12 genes, and it is the primary stewartan EPS biosynthesis cluster (Coplin and Majerczak 1990; Wang 2011). The *cpsA* gene codes for WceG, a glycosyltransferase protein, and the *cpsB* gene codes for Wza, a periplasmic protein involved in export of EPS. A previous study by Thapa et al. (2012) described primers *cpsF* (5'-AAGGTGCCAGCCTCTCTCTG-3') and *cpsR* (5'-ACTATCCTGACTCAGGCACT-3') (Fig. 1) as being designed from the *cpsA* gene and highly selective for *P. stewartii* subsp. *stewartii*. However, in this study, a Clustal Omega alignment of the intergenic sequence between the *cpsA* and *cpsB* genes of *P. stewartii* subsp. *stewartii* reference strain DC283 (GenBank accession number CP017581) and *P. stewartii* subsp. *indologenes* strain LMG2632 (GenBank accession number JPKO01000031) revealed a 182-bp deletion in *P. stewartii* subsp. *indologenes* strain LMG2632 (Fig. 1). The alignment of the *cpsAB* intergenic region (Fig. 1) showed that the *cpsF* and *cpsR* primer binding sites were actually present in both *P. stewartii* subspecies, which suggested that two different-sized amplicons should be generated: 375 bp for *P. stewartii* subsp. *stewartii* and 193 bp for *P. stewartii* subsp. *indologenes*. Thapa et al. (2012) reported no amplification using *cpsF/cpsR* from either of two *P. stewartii* subsp. *indologenes* isolates tested (ATCC 35396 and ATCC 51785).

The deleted region in *P. stewartii* subsp. *indologenes* was targeted in this study for the design of a unique *P. stewartii* subsp. *stewartii*-specific forward primer named *cpsAB2313F* (5'-AGAAAACGCTGATGCCAGAC-3') (Fig. 1). When combined with reverse primer *cpsR*, the expected product is a 256-bp amplicon from *P. stewartii* subsp. *stewartii* DNA and no amplicon from *P. stewartii* subsp. *indologenes* DNA.

Table 1. Pathogenicity, polymerase chain reaction (PCR), and biochemical reactions of bacterial strains used in this study^a

Bacterial strain	Origin (source if known)	Pathogenicity in maize	SW ELISA	Conventional and real-time PCR methods (method I is the real-time PCR assay developed in this study)										Biochemical tests	
				A ^b	B ^c	C ^d	D ^e	E ^f	F ^g	G ^h	H ⁱ	I ^j	Esculin	Indole	
<i>Pantoea stewartii</i> subsp. <i>stewartii</i> strains															
DC283	Nal mutant of SS104 (D. Coplin)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
ES0601	Coix lacryma-jobi, IA, 2006 (C. Block)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
ES9211	Corn, IA, 1992 (C. Block)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
ES9230	Corn, IA, 1992 (C. Block)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
ES9239	Corn, IA, 1992 (C. Block)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
ES9245	Corn, IA, 1992 (C. Block)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
GAL-E8	Avirulent mutant (D. Coplin)	–	+	+	+	+	+	+	+	+	+	+	+	–	–
MU51	Avirulent mutant (D. Coplin)	–	+	+	+	+	+	+	+	+	+	+	+	–	–
SS104	Corn, IL, 1967 (D. Coplin)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
SW1	Corn, OH, 1974 (D. Coplin)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
SW2	Corn, OH, 1974 (D. Coplin)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
SW13	Corn, IN, 1975 (D. Coplin)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
SW14	Corn, IN, 1975 (D. Coplin)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
SW19	Corn, KY, 1975 (D. Coplin)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
SW36	Corn, TN, 1975 (D. Coplin)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
SW39	Corn, NC, 1975 (D. Coplin)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
SW45	Corn, MO, 1975 (D. Coplin)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
SW87-2	Corn, IA, 1987 (G. Lamka)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
SW87-5	Corn, IA, 1987 (G. Lamka)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
SW87-8	Corn, IA, 1987 (G. Lamka)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
SW87-10	Corn, IA, 1987 (G. Lamka)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
SW87-11	Corn, IA, 1987 (G. Lamka)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
SSB30	Corn, IA, 1992 (C. Block)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
ATCC-8199	Sweet corn IA (E. Lindstrom)	+	+	+	+	+	+	+	+	NT	NT	+	–	–	–
ATCC-29231	Insect, CT (D. Sands)	+	+	+	+	+	+	+	+	+	NT	+	–	–	–
NRRL B-195	Corn, WI (A. Riker)	+	+	+	+	+	+	+	+	+	+	+	+	–	–
<i>Pantoea stewartii</i> subsp. <i>indologenes</i> strains															
0696-16	Sudangrass, CA, 1996 (H. Azad)	–	–	+	+	+ ^k	+ ^k	+	+	+	+	+	–	+	–
0696-18	Sudangrass, CA, 1996 (H. Azad)	–	–	+	+	+	–	+	+	+	+	+	–	+	+
0696-19	Sudangrass, CA, 1996 (H. Azad)	–	–	+	+	+	–	+	+	+	+	+	–	+	+
0696-21	Sudangrass, CA, 1996 (H. Azad)	–	–	+	+	+	–	+	+	+	+	+	–	+	+
NRRL B-1061	Sugarcane, FL (B. Bourne)	–	–	+ ^k	+ ^k	+ ^k	+	+	+	+	+	+	–	+	+
ATCC-35397	Melon, Venezuela (J. Wells)	–	–	+ ^k	+ ^k	+ ^k	+	+	+	+	+	+	–	+	+
ATCC-51785	<i>Setaria</i> spp., India	–	–	+ ^k	+ ^k	+ ^k	+	+	+	+	+	+	–	+	+
645-3	Corn seed, P. Rico, 2010 (C. Block)	–	+	+	+	+ ^k	+ ^k	+	+	+ ^k	+	–	+	+	+
645-10	Corn seed, P. Rico, 2010 (C. Block)	–	+	+	+	+ ^k	+ ^k	+	+	+ ^k	+	–	+	+	+
645-14	Corn seed, P. Rico, 2010 (C. Block)	–	+	+	–	+ ^k	+ ^k	+	+	+ ^k	+	–	+	+	+
649-2	Corn seed, P. Rico, 2010 (C. Block)	–	+	+	+	+	+ ^k	+	+	+	+	NT	–	+	+
651-3	Corn seed, P. Rico, 2010 (C. Block)	–	–	+	+	+ ^k	+ ^k	+	+	+	+	+	–	+	+
655-2B-Y1	Corn seed, P. Rico, 2010 (C. Block)	–	+	+	+	+ ^k	+	+	+	+	+	+	–	+	+

(Continued on next page)

^a ELISA, enzyme-linked immunosorbent assay; NT, not tested; *Pseud. syr.*, *Pseudomonas syringae*; P. Rico, Puerto Rico; Sorg., sorghum; SW, Stewart's wilt; *Xant.*, *Xanthomonas*; ISU, Iowa State University; +, positive; –, negative; +/–, ambiguous results.

^b Conventional PCR using CPSL1 and CPSR2c primer set (Coplin et al. 2002).

^c Conventional PCR using HRP1d and HRP3c primer set (Coplin et al. 2002).

^d Conventional PCR using ES16 and ESIG2c primer set (Coplin et al. 2002).

^e Conventional PCR using ESIG1 and ESIG2c primer set (Coplin et al. 2002).

^f Real-time PCR using cps-RT74F and cps-177R primer set and cps-133 probe (Tambong et al. 2008).

^g Conventional PCR using 356 and 341 primer set (Wensing et al. 2010).

^h Conventional PCR using 383 and 385 primer set (Wensing et al. 2010).

ⁱ Real-time PCR using 340 and 354 primer set and 353 probe (Wensing et al. 2010).

^j Real-time SYBR Green PCR with primers cpsAB2313F and cpsR (this study).

^k PCR product not of expected size, or multiple bands were present.

SYBR Green real-time PCR of bacterial strains. PCR amplifications of bacterial strains in Table 1 were performed by using 1 µl (25 to 30 ng/µl) of purified genomic DNA, 5 µl of the 2× concentrated master mix of B-R SYBR Green Supermix for iQ Systems (Quanta Biosciences), 0.4 µl of 10 µmol/liter cpsAB2313F and cpsR

primers, and 3.2 µl of PCR-grade water in a total reaction volume of 10 µl. Amplification conditions were 95°C for 3 min and 40 cycles of 95°C for 15 s and 68°C for 45 s followed by a melt curve from 65°C to 95°C in increments of 0.5°C using a CFX96 real-time PCR detection system with CFX Manager version 3.1 software (Bio-Rad Laboratories,

Table 1. (Continued from previous page)

Bacterial strain	Origin (source if known)	Pathogenicity in maize	SW ELISA	Conventional and real-time PCR methods (method I is the real-time PCR assay developed in this study)										Biochemical tests	
				A ^b	B ^c	C ^d	D ^e	E ^f	F ^g	G ^h	H ⁱ	I ^j		Esculin	Indole
681-4	Corn seed, Brazil, 2010 (C. Block)	–	+	–	–	+	+ ^k	+	+	+ ^k	+	–		+	+
508-3	Corn seed, HI, 2011 (C. Block)	–	+	+	+	+	+	+	+	+	+	–		+	–
508-5	Corn seed, HI, 2011 (C. Block)	–	+	+	+	+	+	+	+	+	+	–		+	–
508-7	Corn seed, HI, 2011 (C. Block)	–	+	+	+	+	+	+	+	+	+	NT		+	–
<i>Pantoea agglomerans</i> strains															
PA99-3	Georgia (R. Walcott)	–	–	–	–	–	–	–	–	+ ^k	–	–		+	–
C9-1	Georgia (R. Walcott)	–	–	–	–	–	–	–	–	–	–	–		+	–
Pagg #1	ISU culture collection	–	–	–	–	–	–	–	–	–	+	–		+	–
Pagg #2	ISU culture collection	–	–	–	–	–	–	–	–	–	–	–		+	–
Pagg-F18	Corn, IA (G. Beattie)	–	–	–	–	–	–	–	–	–	–	–		+	–
Pagg-L9	Corn, IA (G. Beattie)	–	–	–	–	–	–	–	–	–	–	–		+	–
CPO-7	Sorg., Mexico 2007 (H. Silva-Rojas)	–	–	–	–	–	–	–	–	–	–	–		+	–
CPO-9	Corn, Mexico 2007 (H. Silva-Rojas)	–	–	–	–	–	–	–	–	–	–	–		+	–
CPO-16	Sorg., Mexico 2007 (H. Silva-Rojas)	–	–	–	–	–	–	–	–	–	–	–		+	–
CPO-36	Corn, Mexico 2007 (H. Silva-Rojas)	–	–	–	–	–	–	–	–	–	–	–		+	–
CPO-41	Corn, Mexico 2007 (H. Silva-Rojas)	–	–	–	–	–	–	–	–	–	–	–		+	–
<i>Pantoea ananatis</i> strains															
DC130	Corn, MO 1976 (S. Walker)	–	–	+	–	–	–	–	–	–	–	–		+	+
ATCC-8366	Insect gut (E. Steinhaus)	–	–	+	–	–	–	+/–	–	+	+	–		+	+
CPO-8	Corn, Mexico 2007 (H. Silva-Rojas)	–	–	+	–	–	–	–	–	–	–	–		+	+
CPO-12B	Corn, Mexico 2007 (H. Silva-Rojas)	–	–	+	–	–	–	–	–	–	–	–		+	+
CPO-27B	Corn, Mexico 2007 (H. Silva-Rojas)	–	–	+	–	–	–	–	–	–	–	–		+	–
CPO-47	Corn, Mexico 2007 (H. Silva-Rojas)	–	–	+	–	–	–	–	–	–	–	–		+	+
CPO-95	Corn, Mexico 2007 (H. Silva-Rojas)	–	–	+	–	–	–	–	–	–	–	–		+	+
PNA97-1	Melon, GA (R. Walcott)	–	–	+	–	–	–	+/–	–	–	–	–		+	+
PNA98-2	Melon, GA (R. Walcott)	–	–	+	–	–	–	–	+	–	–	–		+	+
ATCC-19321	Wheat (D. Pon)	–	–	+ ^k	–	–	–	+	–	+	+	–		+	+
ATCC-31225	S. Lindow	–	–	+ ^k	+	+	–	+	+	+	+	–		+	+
ATCC-35400	Melon, CA (J. Wells)	–	–	+ ^k	+	+	+	–	+ ^k	+ ^k	+	–		+	+/–
NRRL B-133	Wheat (E. Waldee)	–	+	+ ^k	–	+ ^k	+	+	+	+	+	–		+	+/–
Other isolates															
EA-2, <i>Erwinia amylovora</i>	Apple, ISU culture collection	–	–	–	–	–	–	–	–	–	–	–		–	–
ECC1, <i>Pectobacterium carotovorum</i>	Carrot, ISU culture collection	–	–	–	–	–	–	–	–	–	–	–		+	–
ECA1, <i>Pectobacterium atroseptica</i>	Potato, ISU culture collection	–	–	–	–	–	–	–	–	–	–	–		+	–
ECP1, <i>Erwinia cyripedii</i>	Orchid, ISU culture collection	–	–	–	–	–	–	–	–	–	–	–		+	–
AAA1, <i>Acidovorax avenae</i>	Corn, IA, 2008 (C. Block)	+	–	–	–	–	–	–	–	–	–	–		+	–
NE-S1, <i>Xant. vasicola</i> pv. <i>vasculorum</i>	Corn, NE, 2016 (C. Block)	+	–	+	–	–	–	–	–	–	–	–		+	–
005B, <i>Pseud. syr.</i> pv. <i>syringae</i>	Corn, NE, 2016 (C. Block)	+	–	+ ^k	–	–	–	–	–	+ ^k	–	–		+	–

Inc.). A nontemplate control (NTC) of PCR-grade water was included in all runs.

Standard curves. Standard curves were generated with 10-fold serial dilutions of genomic DNA (1 pg to 10 ng) and 10-fold serially diluted cell suspensions (10^4 to 10^8 CFU/ml) of *P. stewartii* subsp. *stewartii* strain DC283. The experiment was repeated three times.

Specificity and sensitivity of real-time PCR. The specificity of the assay was determined by using 25 to 30 ng of purified genomic DNA from 26 isolates of *P. stewartii* subsp. *stewartii*, 17 isolates of *P. stewartii* subsp. *indologenes*, 11 isolates of *P. agglomerans*, 13 isolates of *P. ananatis*, and 7 non-*Pantoea* isolates (Table 1). Sensitivity was determined with serial 10-fold dilutions of purified genomic DNA of *P. stewartii* subsp. *stewartii* strain DC283 ranging from 1 fg to 10 ng. A second 10-fold dilution series (10^2 to 10^8 CFU/ml) of cell suspension of freshly cultured *P. stewartii* subsp. *stewartii* strain DC283 was prepared in autoclaved milliQ water and adjusted to an initial optical density measured at 600 nm (OD_{600}) of 0.2. Repeatability of the assay was evaluated by testing the samples in duplicate (or triplicate) and in two independent runs.

Statistical analysis. PCR amplification efficiency was calculated from slopes of standard curves using the formula $E = 100 \times [(10^{-1/\text{slope}}) - 1]$. An *F* test from one-way analysis of variance was used to test for significance of difference among the slopes

of standard curves generated from real-time PCR on serial dilutions of *P. stewartii* subsp. *stewartii* strain DC283 cell suspension prepared in the matrix of water or nonconcentrated seed wash. Differences were considered significant at $P \leq 0.05$. The coefficient of variation (CV) was calculated by using the following formula: standard deviation/mean $\times 100$, where the standard deviation is the root mean square deviation of the cycle threshold (Ct) values from their arithmetic mean.

Evaluation of potential inhibitors in seed wash extracts on real-time PCR. A 10-fold dilution series was prepared by diluting freshly cultured *P. stewartii* subsp. *stewartii* strain DC283 cell suspension (adjusted to an initial OD_{600} of 0.2) in autoclaved milliQ water. Another 10-fold dilution series was prepared by diluting the same cell suspension in nonconcentrated seed wash from Stewart's wilt-negative corn accession Ames 23469 02ncai01. The Stewart's wilt-negative status of corn accession Ames 23469 02ncai01 was confirmed by a negative field observation (no disease symptoms on the seed parent plants) and a negative ELISA seed assay. PCR amplification was performed by using 1 μ l of each dilution in a total reaction volume of 10 μ l; it was run under the following conditions: 95°C for 10 min, 45 cycles of 95°C for 10 s and 68°C for 45 s, and melting curve of 65°C to 95°C with increments of 0.5°C using a CFX96 real-time PCR system (Bio-Rad Laboratories, Inc.). Each of the five

Table 2. Real-time polymerase chain reaction (PCR) assay of seed harvested from *Pantoea stewartii* subsp. *stewartii*-infected plants using cpsAB2313F and cpsR primers^a

Corn accessions	Stewart's wilt ELISA on seeds ^b	Real-time PCR, ^c mean Ct $\pm$ SD	
		Concentrated seed wash	Nonconcentrated seed wash
PI 550532 99ncai01	POS	19.70 $\pm$ 0.09	26.58 $\pm$ 0.57
Ames 27025 05ncai01	POS	21.66 $\pm$ 0.21	26.47 $\pm$ 0.18
PI 658075 09ncao01	POS	23.76 $\pm$ 0.47	26.90 $\pm$ 0.02
PI 550481 99ncai01	POS	27.00 $\pm$ 0.69	32.90 $\pm$ 0.47
PI 607512 99ncab01	POS	27.39 $\pm$ 0.42	35.40 $\pm$ 0.0
PI 658081 09ncao01	POS	27.41 $\pm$ 0.49	34.72 $\pm$ 1.27
Ames 25222 99ncai01	POS	27.44 $\pm$ 0.0	30.53 $\pm$ 0.06
PI 595367 00ncai01	POS	27.65 $\pm$ 0.20	32.26 $\pm$ 0.49
Ames 14112 99ncai01	POS	28.17 $\pm$ 0.32	35.50 $\pm$ 1.32
PI 479129 99ncai01	POS	28.60 $\pm$ 0.39	34.13 $\pm$ 0.04
PI 608768 98ncai01	POS	28.70 $\pm$ 0.30	33.39 $\pm$ 0.19
PI 180166 92ncab01	POS	29.24 $\pm$ 0.36	32.95 $\pm$ 0.06
PI 658078 09ncao01	POS	29.42 $\pm$ 0.0	34.29 $\pm$ 0.33
PI 587140 92ncai01	POS	29.80 $\pm$ 0.22	36.08 $\pm$ 0.82
NSL 65865 99ncai01	POS	30.26 $\pm$ 0.10	34.38 $\pm$ 0.45
Ames 27087 04ncai01	POS	30.26 $\pm$ 0.74	35.35 $\pm$ 0.28
PI 586701 06ncai01	POS	30.26 $\pm$ 0.93	33.94 $\pm$ 0.84
Ames 19313 92ncai01	POS	30.76 $\pm$ 0.30	38.20 $\pm$ 0.20
PI 542778 99ncai01	POS	30.76 $\pm$ 0.36	38.30 $\pm$ 0.11
PI 594077 00ncai01	POS	32.96 $\pm$ 0.35	37.78 $\pm$ 0.35
PI 311249 00ncai01	POS	32.96 $\pm$ 1.12	35.24 $\pm$ 0.48
PI 614142 99ncai01	POS	33.60 $\pm$ 0.11	38.99 $\pm$ 0.25
PI 479197 99ncai01	POS	34.14 $\pm$ 0.55	36.16 $\pm$ 1.32
PI 531495 06ncai01	POS	35.34 $\pm$ 0.19	39.54 $\pm$ 0.63
PI 639502 03ncai01	POS	36.44 $\pm$ 1.87	N/A
Ames 22039 04ncai01	POS	36.97 $\pm$ 0.22	N/A
PI 479150 99ncai01	POS	38.17 $\pm$ 1.18	43.83
PI 594051 00ncai01	POS	41.42 $\pm$ 0.79	N/A
PI 600755 98ncai01	POS	43.45 $\pm$ 0.0	N/A
PI 232963 04ncai01	POS	N/A	N/A
PI 597927 04ncai02	POS	N/A	N/A
Negative control lots			
Ames 27185 15ncai01	NEG	N/A	N/A
Ames 28954 08ncai01	NEG	N/A	N/A
PI 221846 00ncai01	NEG	N/A	N/A
PI 548802 12ncai01	NEG	N/A	N/A
PI 558533 92ncai01	NEG	N/A	N/A

^a N/A, not applicable (no observed Ct value); NEG, no detection; POS, positive detection of *P. stewartii*; SD, standard deviation.

^b Enzyme-linked immunosorbent assay (ELISA) tests were performed according to the manufacturer's recommendations (Agdia Inc.).

^c Samples were considered positive for *P. stewartii* subsp. *stewartii* when mean cycle threshold (Ct) value was <40 of 45 amplification cycles (values are means of two replicates).

dilutions (ranging from 10^4 to 10^8 CFU/ml) spiked in the background solution of water or nonconcentrated seed wash was run in triplicate, and the experiment was conducted twice. Standard curves were generated by plotting Ct values versus $\log_{10}$ CFU per milliliter cell

suspension spiked in water or seed wash, and slopes of the linear regression lines were compared by statistical analysis.

SYBR Green real-time PCR on seed wash extracts from corn seed. Fifty-seven corn seed lots were tested by the *P. stewartii*

Table 3. Real-time polymerase chain reaction (PCR) with cpsAB2313F and cpsR primer pair on corn seed lots of tropical or subtropical origin that tested positive with the Stewart's wilt enzyme-linked immunosorbent assay (ELISA)^a

Corn seed lots	Seed production location	Stewart's wilt symptoms on parent plants in the field	Stewart's wilt ELISA on seeds ^b	Real-time PCR on seed wash ^c	Conventional PCR product size, bp
A-28655 (Commercial)	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193
A-28653 (Commercial)	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193
A-28651 (Commercial)	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193
A-28650 (Commercial)	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193
A-28649 (Commercial)	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193
A-28645 (Commercial)	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193
Ames 32321 16ncei01	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193
Ames 32327 15ncei01	St. Croix, U.S.A.	NEG	POS	NEG (Ct = N/A)	193
PI 478954 15ncei01	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193
PI 494936 15ncei01	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193
PI 503900 15ncei01	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193
PI 511656 15ncei01	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193
PI 593012 10ncei01	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193
PI 641239 12ncei01	St. Croix, U.S.A.	NEG	POS	NEG (Ct = N/A)	193
PI 641245 10ncei01	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193
PI 331452 14ncei01	Hawaii	NEG	POS	NEG (Ct = N/A)	193
PI 498644 15ncei01	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193
PI 628461 15ncei01	Puerto Rico	NEG	POS	NEG (Ct = N/A)	193

^a Ct, cycle threshold value from two replicates; N/A, no observed Ct value after 45 amplification cycles; NEG, no detection of *Pantoea stewartii* subsp. *stewartii*; POS, false positive detection of *P. stewartii*.

^b ELISA reagents were obtained from Agdia Inc., and the test was performed according to the manufacturer's recommendations.

^c Both nonconcentrated and concentrated seed wash (as explained in the Materials and Methods section) were tested by real-time PCR with cpsAB2313F and cpsR primer pair.

Table 4. Comparison of extraction procedures for real-time polymerase chain reaction (PCR) detection of *Pantoea stewartii* subsp. *stewartii* in corn seeds^a

SW-positive seed lots ^b	Real-time PCR, mean Ct ± SD						Seeds ground in water or 1× PBS (nonconcentrated seed wash) ^d
	Whole seeds ^c						
	1× PBS		Saline (0.85% NaCl) with 0.02% Tween-20		Deionized (milliQ) water		
	Nonconcentrated seed wash ^d	Concentrated seed wash	Nonconcentrated seed wash ^d	Concentrated seed wash	Nonconcentrated seed wash ^d	Concentrated seed wash	
Seed lot LN1(3.5% kernel infection)	N/A	37.30 ± 1.97	N/A	28.00 ± 0.37	N/A	32.41 ± 1.44	N/A
Seed lot AN1 (9% kernel infection)	N/A	36.28 ± 1.30	N/A	36.48 ± 0.78	33.7 ± 0.39	27.10 ± 0.24	N/A
Seed lot AR4 (37% kernel infection)	N/A	27.10 ± 0.64	N/A	21.71 ± 0.01	29.55 ± 0.37	23.77 ± 0.37	N/A

^a Ct, cycle threshold (values represent the mean of two replicates); N/A, not applicable (there was no observed Ct value during the 45 amplification cycles); SD, standard deviation; SW, Stewart's wilt.

^b Seed lot AR4 was harvested from *P. stewartii* ssp. *stewartii*-inoculated dent corn inbred A632Ht, and seed lots LN1 and AN1 were harvested from naturally infected A632Ht plants.

^c Untreated whole seeds were processed as described in the Materials and Methods section.

^d Ground seed mixtures in sterile 1 $\times$ phosphate buffered saline (PBS), pH 7.4, or water were processed as described in the Materials and Methods section.

ELISA (Agdia Inc.) and SYBR Green real-time PCR. This included 31 seed lots (Table 2) harvested from known *P. stewartii* subsp. *stewartii*-infected field plants (infection verified during phytosanitary field inspection), 5 negative control seed lots from noninfected field plots (Table 2), and 18 seed lots from Puerto Rico, St. Croix, and Hawaii that showed no Stewart's wilt symptoms in field inspections but seed tested positive by ELISA (Table 3).

Fifty untreated whole corn seeds per seed lot were incubated with 50 ml of autoclaved milliQ water in sterile flasks at room temperature on a shaker at 160 rpm for 4 h. A 1,200- μ l aliquot was removed and

centrifuged at 1,000 RCF for 3 min to pellet corn debris; then, 1,100 μ l of the supernatant was transferred to a new tube. Direct PCR amplification of the nonconcentrated water extracts was performed by using 2 μ l of supernatant, 5 μ l of the 2 $\times$ concentrated master mix of B-R SYBR Green Supermix for iQ Systems (Quanta Biosciences), 0.4 μ l of 10 μ mol/liter of each of the cpsAB2313F and cpsR primers, and 2.2 μ l of PCR-grade water in a total reaction volume of 10 μ l under the conditions 95°C for 10 min, 45 cycles of 95°C for 10 s and 68°C for 45 s, and melting curve of 65°C to 95°C with increments of 0.5°C using a CFX96 real-time PCR detection

cpsA gene
stop codon

DC283 GCCATTTTGTTCAAAACCGTCGGTGTCGTGCTTAAGCGTGATGGTGCTTATTAAACCGCAG
LMG2632 GCCATTTTGTTCAAAACCGTCGGTGTCGTGCTTAAGCGTGATGGTGCTTATTAAACCGCAG

cpsF

DC283 GAAAAAGGTGCCAGCCTCTCTCTGACAGCACACGACAGGGGAGCGGGTTGGTACATTTTT
LMG2632 GAAAAAGGTGCCAGCCTCTCTCTGACAGCACACGACAGGGGAGCGGGTTGGTACATTTTT

DC283 CTAAAAAAAGCGGCTTTGTTGAATAATAGGTAATCATTCTGTTTTGTCTGCACTGTTTT
LMG2632 CTAAAAAA-----

cpsAB2313F

DC283 GGTAGAAAACGCTGATGCCAGACAGAACACCGTCATACGCAAGCATGTAATGCATTGATT
LMG2632 -----

DC283 TTATGTTATCTGAAACAACCGTTTTTCATAGTACCACAGCATAAAATCCATTTATTCAACA
LMG2632 -----

DC283 AAGCCAAAAAAGCGGTACGGCATTGATAAACAGCAATGTTCTGCTTTGCTTTATTTATT
LMG2632 -----AAGCGGTACGGCATTGATAAACAGCCATGTTCTGCTTTGCTTTATTTCTT

DC283 TCTGGGTAAAGCGCGTGATCGGTAAAAGACAGGAGCTAAGAATAATGAACGACATGTTCA
LMG2632 TCTGGGTAAAGCGCGTGATCGGTAAAAGACAGGAGCTAAGAATAATGAACGACATGTTCA

cpsR

DC283 GTGCCTGAGTCAGGATAGTTTTACTTTTTTTGAGCGCTTACACAGCGGCGTACTTGCCTCT
LMG2632 GTGCCTGAGTCAGGATAGTTTTACTTTTTTTGAGCGCTTACACAGCGGCGTACTTGCCTCT

DC283 ATCAAACATTAAGTATAGCGAAGATCTAAATGATTACAATGAAAATGAAGATGATACCT
LMG2632 ATCAAACATTAAGTATAGCGAAGATCTAAATGATTACAATGAAAATGAAGATGATACCT

cpsB gene
start codon

Fig. 1. Alignment of intergenic nucleotide sequence between the *cpsA* and *cpsB* genes of *Pantoea stewartii* subsp. *stewartii* strain DC283 (GenBank accession number CP017581) with *Pantoea stewartii* subsp. *indologenes* strain LMG2632 (GenBank accession number JPK001000031) using Clustal Omega. Dashes indicate the 182-bp sequence deletion in strain LMG2632. The stop codon of *cpsA* gene and the start codon of *cpsB* gene are highlighted. Primer binding site sequences are in bold and underlined with primer names above the sequence.

system with CFX Manager version 3.1 software (Bio-Rad Laboratories, Inc.). An NTC using PCR-grade water was included in all runs to monitor contamination.

For the concentrated seed extracts, after the same 3-min centrifugation at 1,000 RCF to pellet corn debris, the supernatant was transferred to a new tube and further centrifuged at 13,000 RCF for 10 min to pellet any bacteria. The supernatant was completely removed with a pipette, and 20 μ l of TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer was added to the pellet. The tube was vortexed for 10 s and heated in a water bath at 95°C for 10 min to disrupt the bacterial cells. After cooling for 5 min, the tubes were centrifuged at 13,000 RCF for 3 min to pellet any cell debris, and the supernatant containing DNA was transferred to a new tube; 2 μ l of this DNA suspension was used for real-time PCR. Amplifications were performed with the same protocol described for the nonconcentrated seed wash, except for initial denaturation at 95°C for 3 min.

Because several bacterial species, including those in genera *Pantoea*, *Pseudomonas*, *Burkholderia*, *Paenibacillus*, *Enterobacter*, *Acetivibacter*, and *Sphingomonas*, have been reported to be present on or inside corn seeds (Johnston-Monje and Raizada 2011; Liu et al. 2012) as part of resident bacterial community, a separate real-time PCR reaction targeting the 16S ribosomal RNA gene sequence was performed with the universal bacterial primers 968F (5'-GAACGC GAAGAACCCTTAC-3') (Lane 1991) and 1492R (5'-TGACTGACT GAGGYTACCCTGTGTTACGACTT-3') (Nübel et al. 1996) to check for PCR inhibition in the concentrated seed wash extracts. Extracts from 25 of the 57 seed lots were tested with the universal bacterial primers, including 16 lots of tropical/subtropical origin, 5 *P. stewartii* subsp. *stewartii*-negative control seed lots, and 4 *P. stewartii* subsp. *stewartii*-positive control seed lots. PCR amplifications were performed with 2 μ l of DNA suspension from the concentrated seed wash, 5 μ l of the 2 $\times$ master mix of B-R SYBR Green Supermix for iQ Systems (Quanta Biosciences), 0.4 μ l of 10 μ mol liter⁻¹ 968F and 1492R primers, and 2.2 μ l of PCR-grade water in a total reaction volume of 10 μ l under the following conditions: 95°C for 3 min; 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min; and melting curve of 65°C to 95°C with increments of 0.5°C using a CFX96 real-time PCR system (Bio-Rad Laboratories, Inc.).

Conventional PCR. If a seed lot tested positive by the Stewart's wilt ELISA and negative by the *P. stewartii* subsp. *stewartii*-specific real-time PCR, a conventional PCR was performed with primer pair cpsF/cpsR (Thapa et al. 2012). The presence of a single 193-bp amplicon indicated the presence of *P. stewartii* subsp. *indologenes* and confirmed the absence of *P. stewartii* subsp. *stewartii* (375-bp amplicon expected). Conventional PCR was carried out with 2 μ l of DNA suspension from the concentrated seed wash or 5 μ l of nonconcentrated water extract, 7.5 μ l of 2 $\times$ concentrated GoTaq Green master mix (Promega), and 0.5 μ l of 10 μ mol/liter each primer in a total reaction volume of 15 μ l under the following conditions: 95°C for 3 min and 40 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 1 min using the MyCycler thermal cycler (Bio-Rad Laboratories, Inc.). PCR products were run on a 2% agarose gel containing GelRed stain (Biotium Inc.).

Sanger sequencing of PCR products. PCR products generated from real-time and conventional PCR were purified with a PCR clean-up/gel extraction kit (IBI Scientific) and sequenced using the cpsR reverse primer at the Iowa State University DNA Sequencing Facility.

Results

Evaluation of ELISA and PCR methods for detection of *P. stewartii* subsp. *stewartii*. All 26 *P. stewartii* subsp. *stewartii* isolates were pathogenic on Stewart's wilt-susceptible corn inbred Mt42 seedlings, except for two known avirulent isolates GalE8 and Mu51 (Coplin et al. 2002). Little variation was seen among isolates, because they all tested positive by ELISA, were amplified by all nine PCR primer sets, and were negative for esculin and indole reactions (Table 1). The 11 *P. agglomerans* isolates were nonpathogenic on Mt42 corn, ELISA negative, and PCR negative on assays A to I

except for isolate PA99-3, which was positive on assay G, and Page#1, which was positive on assay H. All 11 *P. agglomerans* isolates were positive for esculin hydrolysis and negative for indole reaction (Table 1). The *P. ananatis* isolates were nonpathogenic on corn, ELISA negative (except for a weak positive reaction with NRRL B-133), esculin positive, and mostly positive for indole reaction (three indeterminate reactions). PCR assay A (Coplin et al. 2002), which targets the *cpsD* gene, produced amplicons from all 10 *P. ananatis* isolates. Most of the *P. ananatis* isolates were PCR negative on assays B to I; however, isolate NRL B-133 and three ATCC isolates (19321, 31225, and 35400) produced amplicons with multiple primer sets (Table 1), although not always of the expected amplicon size.

A total of 17 (10 from corn seed and 7 from other hosts) *P. stewartii* subsp. *indologenes* isolates were tested. The 10 corn strains (645-3, 645-10, 645-14, 649-2, 651-3, 655-2B-Y1, 681-4, 508-3, 508-5, and 508-7) represented corn seed lots from Brazil, Hawaii, and Puerto Rico. All 17 *P. stewartii* subsp. *indologenes* isolates were nonpathogenic on Mt42 corn and esculin positive; 14 of 17 were indole positive, with exceptions for isolate 0696-16 from sorghum and isolates 508-3, 508-5, and 508-7 from Hawaii-grown corn. Thirteen of 17 were motile (data not shown), whereas all 26 *P. stewartii* subsp. *stewartii* were nonmotile. Interestingly, all 7 isolates from nonmaize hosts were ELISA negative, but 9 of the 10 isolates from corn seed tested positive by ELISA. All *P. stewartii* subsp. *indologenes* isolates yielded PCR products from at least six of the nine PCR assays (Table 1), although the amplicons were not always of the expected size or multiple PCR products were observed. The only consistently negative assay for the *P. stewartii* subsp. *indologenes* isolates was assay I, which was developed in this study.

In summary, although ELISA and the nine tested PCR primer sets were found to be effective for identifying *P. stewartii* subsp. *stewartii*, sets A to H frequently yielded false positive results with *P. stewartii* subsp. *indologenes* isolates and gave scattered positive reactions from *P. ananatis* and occasional *P. agglomerans* isolates (Table 1). The indole test performed reasonably well in helping to separate *P. stewartii* subsp. *stewartii* strains (indole negative) from *P. stewartii* subsp. *indologenes* (most were indole positive). The esculin hydrolysis test was even better; it was 100% effective in distinguishing *P. stewartii* subsp. *stewartii* (esculin negative) from *P. stewartii* subsp. *indologenes* (esculin positive) strains.

Comparison of extraction procedures. Three matrices (PBS buffer, saline plus Tween, and deionized water) were compared for their efficiency in extracting *P. stewartii* subsp. *stewartii* from corn seeds. When Ct values for nonconcentrated seed washes (direct PCR from seed wash extracts) were compared, no amplification occurred from the PBS and saline seed wash extracts (mean Ct = not applicable [N/A]) (Table 4), but two of the three water extracts (AN1 and AR4) were positive. In the concentrated seed wash extracts (high-speed centrifugation and resuspension of pellet), all three seed lots were positive (Table 4), although the sensitivity of detection varied among the extraction matrices. The Ct values for water extracts were three to nine cycles lower (more sensitive) than the corresponding PBS extracts. Two of the three saline extracts (LN1 and AR4) actually had lower mean Ct values than their corresponding water extracts, but water extracts were generally superior for direct detection from nonconcentrated seed washes.

With ground seeds, whether incubated in sterile PBS or water and used directly in real-time PCR, no amplification (mean Ct = N/A) was observed for any of the three seed lots (Table 4). Seed components, such as polysaccharides, polyphenols, alkaloids, or tannins, present in the ground seed extracts may have inhibited PCR amplification.

Standard curves. A standard curve of mean Ct value versus the log₁₀ concentration of DC283 genomic DNA (1 pg to 10 ng) showed a linear correlation ($R^2 = 0.998$, slope = -3.58) and a PCR efficiency of 90.25% (Fig. 2A). The standard curve of Ct value versus bacterial cell concentration (10^4 to 10^8 CFU/ml) showed a linear correlation ($R^2 = 0.993$, slope = -3.483) with a PCR efficiency of 93.69% (Fig. 2B).

Specificity, sensitivity, and repeatability of SYBR Green real-time PCR. Diagnostic specificity of the assay was 100% for the 26 *P. stewartii* subsp. *stewartii* strains tested (Table 1). All strains showed positive amplification with a melting temperature of 80.5°C

Fig. 2. Standard curves of real-time polymerase chain reaction assay with cycle threshold (Ct) values plotted against **A**, 10-fold serial dilutions of *Pantoea stewartii* subsp. *stewartii* strain DC283 purified genomic DNA starting with 10 ng, **B**, bacterial cells spiked in nuclease free water starting with 10^8 CFU/ml, and **C**, bacterial cells spiked in seed wash from Stewart's wilt (SW)-negative corn seeds starting with 10^8 CFU/ml. Values represent mean Ct from a total of three replicates (three independent runs) for genomic DNA and six replicates (two runs with three replicates each) for bacterial cells spiked in water or seed wash. Error bars represent standard deviation. Linear regression equations ($y = a + bx$) are displayed on the graphs. R^2 indicates coefficient of correlation.

(Fig. 3 A and B). Agarose gel electrophoresis confirmed the presence of an amplicon of the expected 256-bp size. The mean Ct values ranged from 13.11 ± 0.6 to 18.95 ± 0.5 for the *P. stewartii* subsp. *stewartii* strains (data not shown). No amplification (Ct = N/A) was observed for all other *Pantoea* spp., including 16 isolates of *P. stewartii* subsp. *indologenes*, 11 isolates of *P. agglomerans*, and 13 isolates of *P. ananatis*. All seven non-*Pantoea* isolates also tested negative (mean Ct = N/A).

The minimum detection level calculated from the standard curves was ~ 0.1 pg for genomic DNA (17 genome equivalents per reaction; genome size = 5,233,214 bp) with a mean Ct value of 35.11 and 2.6 CFU per reaction for bacterial cell suspension (Fig. 2 A and B).

Repeatability of the real-time PCR assay was evaluated by testing five serial 10-fold dilutions of strain DC283 cell suspension (10^4 to 10^8 CFU/ml) spiked in water in two independent real-time PCR runs with three replicates per run. The within-run CV was $<2.4\%$, whereas the between-run CV was $<2.8\%$.

Evaluation of potential inhibitors in seed wash extracts on real-time PCR. Standard curves showed a linear correlation between Ct values and $\log_{10}$ bacterial cells mixed into water ($R^2 = 0.993$, slope = -3.483 , PCR efficiency = 93.69%) (Fig. 2B) or mixed with negative corn seed wash water extract ($R^2 = 0.993$, slope = -3.536 , PCR efficiency = 91.78%) (Fig. 2C). No significant difference (P value = 0.84) was found between the slopes of two standard curves, indicating little or no inhibition owing to seed constituents in the seed wash.

Corn seed lots that tested negative with the *P. stewartii* subsp. *stewartii*-specific real-time PCR assay (Tables 2 and 3) still tested positive for the 16S ribosomal DNA amplicon (~ 524 bp) with universal bacterial primer set 968F and 1492R (Supplementary Table S1), having a melt temperature of $86.5 \pm 0.5^\circ\text{C}$. The results confirmed that bacteria other than *P. stewartii* subsp. *stewartii* were present in the samples and amplifiable under the test conditions. No false negative results were observed.

SYBR Green real-time PCR on seed wash extracts from corn seed. The PCR results for seed wash extracts of 31 Stewart's wilt-infected corn seed lots are shown in Table 2. Twenty-five of the 31 nonconcentrated seed wash samples tested positive (Table 2) with mean Ct values from 26.47 to 43.83 (45 cycles) (Fig. 3C), indicating a diagnostic sensitivity of 80.6%. Six seed lots did not produce a fluorescent signal (Ct = N/A), and one had a weak Ct value of 43.83 that would likely be considered negative from an unknown sample. The melt curve temperature of $80 \pm 0.5^\circ\text{C}$ was consistent across seed lots (Fig. 3D). Agarose gel electrophoresis (data not shown) confirmed the presence of the 256-bp band. When DNA suspensions from concentrated seed washes were used, diagnostic sensitivity improved to 93.5% (29 of 31 seed lots), with mean Ct values from 19.7 to 43.45 (Table 2). Two seed lots did not produce a fluorescent signal (Ct = N/A), and two had weak Ct values >40 that would likely be considered negative if they were unknowns. On average, there was an improvement in sensitivity of about five Ct cycles between the nonconcentrated seed extracts and the concentrated seed extracts (Table 2).

Seed lots from five ELISA-negative corn accessions tested negative (mean Ct = N/A) from both concentrated and nonconcentrated seed washes (Table 2). The positive control seed lot AR4 (Table 4) tested positive in all runs. Seed lot AN1 (Table 4) was also included, and its amplicon identity was confirmed by sequencing and comparison with the Genbank sequence for DC283. All 18 seed lots from Hawaii, Puerto Rico, and St. Croix with negative field inspections but positive ELISA results were PCR negative (mean Ct = N/A) from both concentrated and nonconcentrated seed wash extracts (Table 3).

Conventional PCR. Pure cultures of 7 strains of *P. stewartii* subsp. *stewartii* and 12 strains of *P. stewartii* subsp. *indologenes* showed size differences of PCR products from conventional PCR using primers cpsF/cpsR, a 375-bp band from *P. stewartii* subsp. *stewartii*, and a 193-bp band from *P. stewartii* subsp. *indologenes* (Fig. 4A). All 10 *P. stewartii* subsp. *indologenes* from corn seed produced the 193-bp amplicon. Bands for 7 of 10 isolates (645-3, 645-14, 649-2, 651-3, 508-3, 655-2B, and 681-4) are shown in Fig. 4A.

Sequencing of the conventional PCR products from *P. stewartii* subsp. *indologenes* isolates 0696-16, 508-3, 681-4, 649-2, and ATCC 35397 had excellent alignment with *P. stewartii* subsp. *indologenes* strain LMG2632 (Fig. 5A).

The absence of *P. stewartii* subsp. *stewartii* from the 18 ELISA false positive seed lots in Table 3 was confirmed by conventional PCR. PCR amplicons from eight Stewart's wilt-positive seed lots and eight false positive seed lots are shown side by side in Fig. 4B. Based on the combination of results from pathogenicity, physiological and biochemical tests, real-time and conventional PCR, and sequencing of *cpsAB* intergenic sequence, the isolates from corn seed lots of tropical origin were confirmed to be *P. stewartii* subsp. *indologenes*.

In addition to the nine PCR methods covered in Table 1, we also evaluated PCR methods by Gehring et al. (2014) and Thapa et al. (2012). The *recA* and *galE* primer pairs of Gehring et al. (2014) were reported to be subspecies specific and capable of distinguishing the two subspecies by SNPs present at the 3' end of primers. This study found that the putative *P. stewartii* subsp. *stewartii*-specific *recA* primers also amplified 4 of 10 *P. stewartii* subsp. *indologenes* corn seed isolates. Three of the *recA*-positive isolates (645-3, 645-10, and 645-14) were of Puerto Rican origin, and one (681-4) was from Brazil. Sequencing of *recA* amplicons indicated that the expected *recA* SNPs were not always present (data not shown). The *P. stewartii* subsp.

stewartii-specific *galE* primers of Gehring et al. (2014) were consistent and amplified only *P. stewartii* subsp. *stewartii*, and the *P. stewartii* subsp. *indologenes*-specific *galE* primers amplified only *P. stewartii* subsp. *indologenes*.

The three primer pairs of Thapa et al. (2012) were not specific for *P. stewartii* subsp. *stewartii*, because they always amplified one (*hrpN*) or two (*hrpN* and *wtsE*) bands from every *P. stewartii* subsp. *indologenes* isolate (Supplementary Fig. S1). Nechwatal et al. (2018) reported that the *hrpN* primers produce a 212-bp amplicon from *P. stewartii* subsp. *indologenes* (a 12-bp insert) compared with the expected 200-bp amplicon from *P. stewartii* subsp. *stewartii*. Thus, the lowermost band of ~200 bp (Thapa et al. 2012) for *P. stewartii* subsp. *indologenes* is probably a mixture of two amplicons (the 193-bp amplicon from the *cpsF/cpsR* primers and a 212-bp *hrpN* gene product), which cannot be separately resolved on a low-percentage agarose gel.

Discussion

The results from the ELISA and nine PCR comparisons added significant new information and supported conclusions from earlier studies (Block et al. 2011; Gehring et al. 2014; Nechwatal et al. 2018; Tambong 2015), which showed that none of the common laboratory assays for *P. stewartii* subsp. *stewartii* were 100% reliable. Although all of the methods could readily detect *P. stewartii* subsp. *stewartii*, they were prone to

Fig. 3. **A and C,** Amplification plots and **B and D,** melt curve profiles of *Pantoea stewartii* subsp. *stewartii*-specific amplicons generated in real-time polymerase chain reaction assay with *cpsAB2313F* and *cpsR* primer pair using **A and B,** purified genomic DNA or **C and D,** seed wash from corn seed. Note the melting temperature at 80.5°C for genomic DNA and 80°C for seed wash. RFU (Y-axis), relative fluorescence units.

false positives, especially from DNA of *P. stewartii* subsp. *indologenes* and even from occasional isolates of *P. ananatis*.

There is high DNA sequence homology between the two *P. stewartii* subspecies at the primer binding sites. Multiple bands or bands of unexpected size were noted from many of the *P. stewartii* subsp. *indologenes* isolates, particularly with assays C and D that use the same reverse primer (Coplin et al. 2002). None of the off-type bands were sequenced, but they suggest possible sequence variability within the *P. stewartii* subsp. *indologenes* genome.

P. stewartii subsp. *indologenes* has been reported on several non-grass hosts and may have a more heterogeneous genome than *P. stewartii* subsp. *stewartii*, which is specialized to corn. The fact that all 7 *P. stewartii* subsp. *indologenes* strains obtained from noncorn hosts were ELISA negative but that 9 of the 10 corn seed isolates were ELISA positive also shows a dissimilarity for the corn isolates. The *galE* primers (Gehring et al. 2014) correctly recognized the respective subspecies in this study, but they depend on single-base pair differences (SNPs) for pathogen identification. The method would require an additional step of sequencing the amplicons to confirm the presence of subspecies-specific *galE* SNPs, because conventional agarose gel electrophoresis is not sufficient to resolve single-base differences. Moreover, the consistent presence of these SNPs has not yet been shown in any study and would need testing of additional *P. stewartii* subsp. *indologenes* isolates derived from corn seed. The SYBR Green real-time PCR developed here exploits a 182-bp missing fragment from the *cpsAB* region of *P. stewartii* subsp. *indologenes*, a fragment that was present in all 26 *P. stewartii* subsp. *stewartii* isolates tested. Sequencing of amplicons from five *P. stewartii* subsp. *indologenes* isolates (Fig. 5A) confirmed the absence of the 182-bp fragment in the *cpsAB* intergenic sequence, which we

believe to be a characteristic of *P. stewartii* subsp. *indologenes*. None of the 36 strains of other *Pantoea* species and 7 non-*Pantoea* species showed a cross-reaction.

The analytical sensitivity for purified DNA of 0.1 pg from real-time PCR was higher than the reported sensitivity of 0.5 pg for multiplex PCR (Thapa et al. 2012) and 1 pg for TaqMan real-time PCR (Tambong et al. 2008). The sensitivity of 2.6 CFU per reaction for bacterial cell suspension was similar to the multiplex PCR (1 CFU per reaction) reported by Thapa et al. (2012) and conventional PCR (2.5 CFU per reaction) by Nechwatal et al. (2018) but more sensitive than that of ELISA (2×10^4 CFU/g) and conventional PCR (20 CFU per reaction) by Coplin et al. (2002) or real-time PCR (10 CFU per reaction) by Tambong et al. (2008).

Concentrating the seed wash by centrifugation led to enrichment of target bacterial cells and an increase in the amount of template used in real-time PCR; hence, it resulted in lower Ct values. The results were not unexpected. However, PCR inhibitors, such as Tween-20 and salts present in the nonwater extraction buffers, could interfere with DNA amplification in centrifuge-only sample preparations. Similar PCR inhibition occurred with ground seed extracts and treated seed. Obviously, extracting and purifying DNA should eliminate problems of PCR inhibition, but the goal was to show that even a quick seed wash and minimal centrifugation were often quite effective in detecting *P. stewartii* subsp. *stewartii*. Ivanoff (1933) and Rand and Cash (1933) reported the presence of *P. stewartii* bacterial cells in the endosperm and chalazal region (kernel base) of infected kernels. Incubating corn seeds with water as an extraction matrix should result in good recovery of externally located bacterial cells.

This is the first study to validate real-time PCR on seed lots harvested from naturally infected plants using seed washes from whole corn seeds

Fig. 4. Conventional polymerase chain reaction (PCR) using *cpsF* and *cpsR* primer pair showing PCR product size differentiation for *Pantoea stewartii* subsp. *stewartii* and *Pantoea stewartii* subsp. *indologenes*. **A**, PCR was performed with purified genomic DNA from *P. stewartii* subsp. *indologenes* strains 0696-21 (lane 1), 0696-19 (lane 2), 645-3 (lane 3), 645-14 (lane 4), 649-2 (lane 5), 651-3 (lane 6), 508-3 (lane 7), 655-2B (lane 8), 681-4 (lane 9), ATCC-51785 (lane 10), ATCC-35397 (lane 11), and NRRL B-1061 (lane 12) and *P. stewartii* subsp. *stewartii* strains NRRL B-195 (lane 13), DC283 (lane 14), SW1 (lane 15), ES9245 (lane 16), SS104 (lane 17), ATCC-29231 (lane 18), and ATCC-8199 (lane 19). Lane M contains an 100-bp DNA ladder. **B**, PCR was performed using 5 μ l of nonconcentrated seed wash from Stewart's wilt enzyme-linked immunosorbent assay (ELISA) and real-time PCR-positive (Table 2) corn seed lots PI 587140 (lane 2), PI 595367 (lane 3), PI 658075 (lane 4), PI 658078 (lane 5), PI 658081 (lane 6), PI 180166 (lane 7), Ames 19313 (lane 8), and Ames 27025 (lane 9) and real-time PCR-negative but ELISA-positive (Table 3) corn seed lots A-28655 (lane 11), A-28651 (lane 12), A-28649 (lane 13), A-28645 (lane 14), Ames 32327 15ncei01 (lane 15), PI 503900 15ncei01 (lane 16), PI 593012 10ncei01 (lane 17), and PI 641239 12ncei01 (lane 18). Lanes 1 and 10 have amplicons from *P. stewartii* subsp. *stewartii* strain DC283 and *P. stewartii* subsp. *indologenes* strain ATCC-35397, respectively. Lane 19 is nontemplate sterile water control; lane M contains a 50-bp DNA ladder.

without requiring pathogen isolation or DNA purification. All but 2 of 31 corn seed lots from naturally infected plants tested positive by SYBR Green real-time PCR. ELISA tests of two 75-kernel subsamples from the two negative seed lots gave a positive absorbance signal from one of two subsamples for each of those two seed lots. Because we used small samples of just 50 seeds, it is possible that an infected kernel

was not present in the subsamples. With 50 seeds from a 1% infected seed lot, there is only a 40% probability of including 1 infected seed in the sample (SeedCalc software, ISTA, <https://www.seedtest.org/en/home.html>). A minimum sample size of 250 seeds would be needed from the 1% infected seed lot to have a confidence level of 90% that at least one infected kernel was included.

Fig. 5. Amplicon sequences generated from **A**, conventional polymerase chain reaction (PCR) using purified genomic DNA of *Pantoea stewartii* subsp. *indologenes* strains 0696-16, ATCC-35397, 508-3, 681-4, and 649-2 and **B**, real-time PCR of seed wash from seed lot AN1. Sequences were aligned with reference sequences of *P. stewartii* subsp. *indologenes* strain LMG2632 (GenBank accession number JPKO01000031) and *Pantoea stewartii* subsp. *stewartii* strain DC283 (GenBank accession number CP017581) using Clustal Omega. The primer binding site for the cpsR primer used in the sequencing reaction is highlighted. *Identical sequence.

Other more complex methods have been developed for *P. stewartii* subspecies differentiation. Wu et al. (2007) used a proteomics-based approach to identify differences in protein expression profiles of the two subspecies. A genotyping method developed by Xu et al. (2010) involves evaluation of a complex DNA fingerprint for subspecies differentiation. Both methods are time consuming and require expensive equipment or specialized expertise, and they do not seem to be practical for routine seed health testing. The multigene oligonucleotide array method described by Tambong (2015) involves multiplex PCR reactions, digoxigenin labeling of amplicons, and probe hybridization, and it does not seem to be a simple, convenient, or cost-effective option.

The *P. stewartii* ELISA developed by Lamka et al. (1991) is still a very effective test and commonly used for phytosanitary seed certification. The SYBR Green real-time PCR method developed here could serve as a standalone method, or it could be used in combination with ELISA. Corn seed lots that test positive by ELISA can be confirmed for the presence or absence of *P. stewartii* subsp. *stewartii* by real-time PCR, conventional PCR, or a combination of both methods. To our knowledge, this is the first study to show PCR methods that can detect *P. stewartii* subsp. *stewartii* directly from the seed wash and readily distinguish it from the closely related *P. stewartii* subsp. *indologenes*.

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