Identification of *Pantoea stewartii* subsp. *stewartii* by PCR and Strain Differentiation by PFGE

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ABSTRACT

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Stewart's bacterial wilt and leaf blight of sweet corn and maize is caused by *Pantoea stewartii* subsp. *stewartii*. This bacterium can be seed transmitted at a low frequency, so it is subject to quarantine restrictions by many countries. To develop a polymerase chain reaction assay for the identification of this pathogen from field samples and for use in seed health tests, four primer pairs were tested. These were selected from the sequences of *hrpS*, *cpsDE*, and the 16S rRNA intergenic transcribed spacer (ITS) region. Under optimal reaction conditions, about 20 and 200 cells of *P. stewartii* could be detected in pure cultures and leaf lesions, respectively. Other plant-associated enteric bacteria (e.g., *P. agglomerans* pv. *herbicola*, *P. ananas*, *Erwinia amylovora*, and *E. carotovora*) either did not produce amplicons or they were not the correct size for *P. stewartii*. To test further for possible false positives, 29 yellow-pigmented bacteria, mainly other *Pantoea* spp., were isolated from lesions on old corn leaves and assayed with the ITS primer sets. Except for weak, variable reactions with three *P. ananas* strains, the bacteria did not test positive. Pulsed field gel electrophoresis (PFGE) was evaluated as an additional test to confirm the identity of *P. stewartii*. After digestion with *SpeI* and *XbaI*, *P. stewartii* strains could be easily distinguished from related *Erwinia* and *Pantoea* spp. and each other.

Additional keywords: *cps*, *Erwinia stewartii*, *hrp*, *Pantoea ananas*, RFLP

Pantoea stewartii subsp. *stewartii* (syn. *Erwinia stewartii* [24] and hereafter referred to as *P. stewartii*) causes Stewart's vascular wilt and leaf blight of sweet corn and maize, a bacterial disease that is responsible for serious crop losses. The disease is spread by the corn flea beetle *Chaetocnema pulicaria* (28), but can be seed transmitted at a very low frequency (6,7,25). However, the potential risk of seed transmission is considered so important in international shipment of corn seed that more than 50 countries ban its import unless it is certified to be free of *P. stewartii*. The traditional techniques for detection and identification of *P. stewartii* include field inspection of plants to be harvested as seed, grow-out tests of seed, and an enzyme-linked immunosorbent assay (ELISA; 22). These assays are time

consuming and relatively insensitive. A polymerase chain reaction (PCR)-coupled ligase chain reaction (LCR) test (30) has been demonstrated to identify the pathogen. However, the use of radioisotopes limits this method for routine assays. A specific, two-step PCR detection method for *P. stewartii* was developed by Blake-more et al. (4). This test uses nested primers complementary to a diagnostic DNA hybridization probe that was generated by arbitrarily primed PCR (5).

There are two major gene clusters that play an important role in the pathogenicity and virulence of *P. stewartii*: (i) the *cps* gene cluster, which is required for production of the exopolysaccharide (EPS) stewartan (13); and (ii) the *hrp* gene cluster, which encodes a type-III secretion system that is necessary for general pathogenicity and the production of water-soaked lesions (10,11,19). Stewartan consists of a seven-monosaccharide repeating unit that contains glucose, galactose, and glucuronic acid. The *cps* gene cluster comprises 12 genes, *cpsA* to *cpsM*, that encode proteins for the assembly and secretion of the repeating units and their polymerization into a macromolecule (13). The genetic organization of the *P. stewartii* *cps* cluster is very similar to that of the *E. amylovora* *ams* gene cluster, which is responsible for the synthesis of amylovoran EPS (3,9). Consequently, amylovoran is very similar in

structure to stewartan (3) and differs in only two sugar residues. These changes are accounted for by three glycosyl-transferase genes that differ between the *ams* and *cps* clusters (14). One of these is *cpsD*. The *hrp* gene cluster of *P. stewartii* is homologous to that of *E. amylovora* (19) and *P. agglomerans* pv. *gypsophilae* (26). It consists of seven complementation groups, plus a linked disease-specific effector region. Gene *hrpS* (formerly *wtsA*) encodes an NtrC-like transcriptional enhancer that is required for the expression of *hrp* secretion and *wts* effector genes and shares significant similarity to the central domains of *P. syringae* pv. *phaseolicola* and *E. amylovora* *hrpS* (19,20).

Pulsed field gel electrophoresis (PFGE) separates large restriction fragments created by digestion of total genomic DNA with endonucleases that cleave infrequently. The patterns generated by PFGE are commonly used to identify clinical pathogens (8), to construct physical genomic maps (23), and to differentiate closely related species, serovars, and pathovars of bacteria (17). For example, this technique has been used to follow the spread of particular strains of *E. amylovora* through Europe and the Mediterranean region (31,32, S. Jock, W.-S. Kim, V. Donat, M. M. Lopez, C. Bazzi, and K. Geider, unpublished data).

In this study, we tested several fast, sensitive, species-specific PCR assays for the detection and identification of *P. stewartii*. Primers were designed to amplify the 16S rRNA intergenic transcribed spacer (ITS) region and two pathogenicity-related genes, *hrpS* and *cpsD*. These assays were evaluated with known strains, typical "false positive" bacteria isolated from corn leaf lesions, and plant material. In addition, we demonstrated the use of PFGE to confirm the identification of *P. stewartii* and to differentiate strains for epidemiological studies.

MATERIAL AND METHODS

Bacterial strains. Reference strains and their sources are listed in Table 1. In addition, *E. mallotivora* CFBP2503, *E.* (syn. *Brenneria*) *quercina* CFBP3617, *E.* (syn. *Brenneria*) *rubrifaciens* CFBP3619, *E. psidii* CFBP3627, *E.* (syn. *Pectobacterium*) *cacticida* CFBP3628, *E.* (syn. *Brenneria*) *salicis* CFBP802, *E. tracheiphila* CFBP2355, *E.* (syn. *Pectobacterium*)

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cyripedii CFBP3613, *E. persicinus* CFBP3622, *E. rhapontici* CFBP3618, *E. nigrifluens* CFBP3616, and *E. amylovora* CFBP1232 were obtained from the Collection Française des Bactéries Phytopathogènes, INRA, Angers, France (Table 2). The identification of all *Pantoea* strains was confirmed and pathogenicity on sweet corn was retested as described below. Strains DC129 and DC130 were received as unidentified isolates from S. Walker (University of Missouri) and later classified as *Pantoea ananas*. Bacteria isolated from Ohio corn leaves collected from 1995 to 1998 were given DC400 strain designations and classified as listed in Table 3.

Isolation and identification of bacteria isolated from maize leaves. Bacteria were isolated from tritirated lesions excised from corn leaves collected in fields in Ohio (Table 3) by plating on casamino acids-peptone-glucose (CPG) agar and phenotypically identified as described in Coplin and Kado (12). Initial identification was based on the gram reaction (KOH test); Hugh-Liefson test; pigment production; sensitivity to stewartan-dependent phage Φ K9 (3); hypersensitive reaction (HR) on tobacco; motility; acid from glycerol,

melibiose, meso-inositol, and sorbitol; and utilization of malonate. Identifications were confirmed by gas chromatographic–fatty acid methyl ester (GC-FAME) analysis with a model HP6890 Microbial Identification System equipped with version 3.9 of the Microbial Identification Aerobic Library (Microbial Identification, Inc., Newark, DE.). Strains were identified to the species and genus levels according to manufacturer's specifications. Strains with similarity indices ≥ 0.3 but lacking the required 0.1 degree of separation between generic classifications were identified as cluster groups. Strains with similarity indices < 0.3 or with identifications of “no match” were considered unclassified. In addition, pathogenicity on sweet corn seedlings was tested as described below.

PCR assay. The primers CPSL1 (CCTGTCAGTCTCGAACC) and CPSR2c (ATCTCGAACCGGTAACC), were designed from the sequence of the *cpsD*-region (14), which encodes synthesis of capsular polysaccharide stewartan and appears to be unique for *P. stewartii* (D. L. Coplin and D. R. Majerczak, *unpublished*). Primers HRP1d (GCACTCATTCGACCAC) and HRP3c (GCGGCATACCTAACC)

are internal to the *hrpS* open reading frame (ORF; 20; Genbank AF282857). Primers from the 16S-23S rRNA/ITS region (EMBL AJ311838) were ES16 (GCGAACTTGGC-AGAGAT, 993 nt in 16S rRNA); ESIG1 (ESR1) (CGAAGC-GAGGACACACG, 71 nt in the ITS region); and ESIG2c (GCGCTTGCCTGT-TATGAG, 361 nt the complementary strand of the ITS region). These positions correspond to nucleotides 993, 1605, and 1895, respectively, in EMBL accession AJ311838.

Whole bacteria were used for a PCR assay that was carried out in a volume of 50 μ l with 25 pmol of each primer and 0.5 U of *Tth* polymerase (Amersham Pharmacia Biotech Europe, Freiburg, Germany) as described previously (1,2). Denaturation was done at 94°C (one cycle for 1 min and subsequent cycles for 15 s); the annealing of primer to the template DNA was for 15 s at 52 to 55°C in the case of the CPS and HRP primers or 55 to 58°C in the case of the ITS primers; and polymerization was at 72°C for 30 s. After 25 cycles (1.5 to 2 h), the PCR product was separated on a 1% agarose gel (1.5 to 2 h at 1.5 V/cm), stained with ethidium bromide, and photographed under UV light.

Table 1. Bacterial reference strains used for polymerase chain reaction assays and pulsed field gel electrophoresis analyses

Strain	Host	Origin	Source (alternate no.)
<i>Pantoea stewartii</i> subsp. <i>stewartii</i> (syn. <i>Erwinia stewartii</i>)			
SS102	<i>Zea mays</i>	New York	16 (Z05)
SS104	<i>Z. mays</i>	Illinois, 1967	ICPPB ^a
SW1, SW2	<i>Z. mays</i>	Ohio, 1974	D. Coplin
SW4, SW18, SW24, SW25, SW28, SW31, SW32	<i>Z. mays</i>	Ohio, 1975	D. Coplin
SW5, SW19 to SW23, SW26	<i>Z. mays</i>	Kentucky, 1975	D. Coplin
SW11	<i>Z. mays</i>	Illinois, 1975	D. Coplin
SW13	<i>Z. mays</i>	Indiana, 1975	D. Coplin
DC117, DC119	<i>Z. mays</i>	Missouri, 1976	S. Walker
DC116, DC122, DC133	<i>Z. mays</i>	Missouri, 1976, avirulent	S. Walker
DC145	<i>Chaetocnema pulicaria</i>	Connecticut, 1976	D. Sands (ATCC29227)
DC146	<i>C. pulicaria</i>	Connecticut, 1976	D. Sands (ATCC29228)
DC147	<i>C. pulicaria</i>	Connecticut, 1976	D. Sands (ATCC29229)
DC162	<i>Z. mays</i>	New York, 1975	T. Woods (Es-5)
DC172	<i>Z. mays</i>	Iowa, 1940, avirulent	Lindstrom (SS11), ICPPB
DC283	<i>Z. mays</i>	Nal ^r mutant of SS104	15
<i>P. ananas</i>			
CU2093	<i>Ananas comosus</i>	Hawaii	A. Alvarez (M189)
DC129, DC130	<i>Z. mays</i>	Missouri, 1976	S. Walker
DC532	...	...	S. Lindow (Cit 30-11)
<i>P. agglomerans</i> pv. <i>gypsophila</i> DC556	<i>Gypsophila paniculata</i>	Israel	I. Barash
<i>P. agglomerans</i> pv. <i>herbicola</i>			
C9-1	<i>Malus sylvestris</i>	United States	C. Ishimaru
Eh358-6A	<i>Prunus domestica</i>	Switzerland, 1975	18
Eh7/95	<i>Z. mays</i>	Germany, 1995	
Eh8/96	<i>Z. mays</i>	Germany, 1996	
EhA1-81	<i>Cotoneaster salicifolius</i>	Switzerland, 1981	18
EhA1-82	<i>C. salicifolius</i>	Germany, 1982	18
EhNZ	<i>Pyrus communis</i> fruits	New Zealand	2
EhSB	<i>P. communis</i>	Germany	18
EhY112-9	<i>M. sylvestris</i>	United States, 1986	18
<i>P. agglomerans</i> subsp. <i>synvitivora</i> Ehs2035	...	England, 1984	18
<i>Pantoea</i> spp. DC131, DC136	<i>Z. mays</i>	Missouri, 1976	S. Walker
<i>Erwinia amylovora</i>			
Ea179	<i>Cotoneaster</i> sp.	Germany, 1979	18
E9	<i>M. sylvestris</i>	USA	18
<i>E. carotovora</i> subsp. <i>carotovora</i> Ecc499	...	Germany, 1986	18
<i>E. carotovora</i> subsp. <i>atroseptica</i> Eca185	...	Germany, 1986	18
<i>Pseudomonas syringae</i> pv. <i>syringae</i> 4U	<i>P. communis</i>	Germany, 1991	W. Zeller
<i>Xanthomonas campestris</i> pv. <i>campestris</i> XCC356	...	Germany	M. Nachtigall

^a ICPPB = International Collection of Plant Pathogenic Bacteria, Davis CA, no longer available.

Pathogenicity tests on corn seedlings.

Sweet corn seedlings (cv. Earliking or Seneca Horizon) were grown at 28°C and 16 h daylight in flats (20 by 10 by 2.5 in.) containing soil:vermiculite:peat moss (1:1:1). In order to assay the virulence of *P. stewartii* strains, 8-day-old seedlings were inoculated using two different inoculation procedures. In the “cut stump” method, 5.0 µl of inoculum (1,250 CFU/plant) were pipetted onto the cut ends of decapitated 8-day-old seedlings; the plants were rated at 10 days after inoculation. For the “whorl” inoculation method, 200 µl of inoculum (10⁷ CFU/ml) in 0.01 M potassium phosphate buffer (pH 7.0) containing 0.2% Tween 40 was placed in the whorls of the seedlings; the plants were rated at 10 days after inoculation as described in Table 3.

Preparation of plant material for PCR. One-week-old seedlings of susceptible sweet corn were inoculated by the cut stump or whorl methods. After 1 week, 10 mg of material from leaves with symptoms or healthy control leaves were cut and slices were placed into an Eppendorf tube containing 100 µl of sterile water. After removing the debris by centrifugation, the suspension was serially diluted and used for the PCR assays as above.

PAGE assay. The PAGE analysis of chromosomal DNA of *P. stewartii* was performed as described by Zhang and Geider (31). Similarity coefficients were calculated from the restriction fragments on the gels according to Nei and Li (27).

RESULTS

Identification of *P. stewartii* by PCR with primers from the rRNA ITS region.

The chromosomal ITS regions encoding the rRNA vary considerably between related bacterial species (21). By comparing the corresponding sequences of the ITS regions from *P. stewartii* (EMBL AJ311838), *E. amylovora* (EMBL AJ010485), *Escherichia coli* (Genbank J01695), and *Erwinia pyrifoliae* (EMBL AJ009930) (Fig. 1A), two primers (ESIG1/ESIG2c) were selected, which appeared to be highly specific for *P. stewartii*. They amplified a 0.29-kb DNA fragment. In order to obtain a larger fragment for easier detection on gels, a second forward primer (ES16) was selected from within the 16S rRNA gene. The ES1G2c/ES16 primers amplified a 0.92-kb fragment. Both primer pairs, ESIG1/ESIG2c and ES16/ESIG2c, were tested with 33 known strains of *P. stewartii*, 12 strains of other *Pantoea* spp., 14 related *Erwinia* spp., and one strain of *Xanthomonas campestris* pv. *campestris* (Table 2). All *P. stewartii* strains yielded the expected amplicons. Only one *P. ananas* strain produced bands with the ES16/IG2 primers and another produced faint bands with both primer sets. Other *Pantoea* and *Erwinia* strains did not produce these PCR products, including the type strains of many present and former *Erwinia* spp. and other plant-associated bacteria (Fig. 2).

Use of the ITS/16S rRNA PCR assay with bacteria isolated from old field

samples. *P. stewartii* is easily isolated from fresh lesions on corn leaves and can be tentatively identified by colony type, odor, and motility and then confirmed with a pathogenicity test (12) or agarose gel electrophoresis of plasmids (15). However, after plant material has been in storage, saprophytic bacteria multiply and *P. stewartii* is harder to recover because it is a poor competitor and cannot easily survive desiccation. The bacteria most frequently isolated are related yellow-pigmented *Pantoea*s, such as *P. agglomerans* pv. *herbicola* or *P. ananas*. To test the validity of our PCR assays using ITS primers, we isolated a number of bacteria from old corn leaves that resembled *P. stewartii* on CPG plates. Corn leaves were collected during the summers of 1995, 1996, and 1998 and held in storage in brown paper bags at 4°C until spring 1999, when we attempted to identify the pathogen among colonies isolated from these leaves on CPG agar. Twenty-seven samples were taken from 24 fields at 13 locations representing seven counties in Ohio. In all, 29 bacterial isolates were characterized by pathogenicity, biochemical traits, and PCR with the ITS primers (Table 3). Seven samples yielded *P. stewartii* and these strains gave the expected amplicons with both primer sets and were sensitive to phage ΦK9, nonmotile, and virulent on sweet corn. The most frequent species was a group of 11 strains that are probably *P. ananas*. Of this group only three gave faint and variable signals with the ITS primer sets. These

Table 2. Polymerase chain reaction (PCR) assays with reference strains of *Pantoea stewartii* subsp. *stewartii* and other bacteria^a

Strain	IG1/IG2c (0.29 kb)	ES16/IG2 (0.92 kb)	HRP (0.9 kb)	CPS (1.1 kb)
<i>Pantoea</i> spp.				
<i>P. stewartii</i> subsp. <i>stewartii</i>				
DC117, DC145, DC172, DC283, SW2, SW4, SW5, SW11, SW19, SS104	+	+	+	+
SW1, SW13, SW18, SW21, SW22, SW23, SW24, SW25, SW26, SW28,				
SW31, SW32, SS102, SS104, DC119, DC146	+	+	nd	nd
DC145	+	±	nd	nd
<i>P. stewartii</i> subsp. <i>stewartii</i> , avirulent strains				
DC122, SW20, DC133, DC147, DC162	+	+	+	+
DC116	+	+	–	–
<i>P. ananas</i>				
CU2093, DC532	–	–	–	–
DC129	–	v	–	–
DC130	v	+	nd	nd
<i>P. ananas</i> pv. <i>uredovora</i> GSPB457 ^b	–	–	nd	–
<i>P. agglomerans</i> pv. <i>herbicola</i>				
Eh7/95	–	–	–	+
Eh C9-1	–	v	–	–
Eh8/96, EhA1-81, EhNZ, EhSB	–	–	–	–
<i>P. agglomerans</i> pv. <i>gypsophylae</i> DC556	–	–	–	–
<i>P. agglomerans</i> subsp. <i>synnitivora</i> Ehs2035	–	–	–	–
<i>Erwinia amylovora</i> Ea1/79, E9	–	–	–	–
<i>Erwinia</i> type strains ^c	–	–	–	–
Other bacteria ^d	–	–	–	–

^a nd = not done; v = PCR reaction giving variable intensity of bands (0 to 30% of normal).

^b GSPB = Göttinger Sammlung Phytopathogener Bakterien, Göttingen, Germany.

^c CFBP = Collection Française des Bactéries Phytopathogènes, INRA, Angers, France: *E. mallotivora* CFBP2503, *E. quercina* CFBP3617, *E. rubrifaciens* CFBP3619, *E. psidii* CFBP3627, *E. cacticida* CFBP3628, *E. salicis* CFBP802, *E. tracheiphila* CFBP2355, *E. cypridii* CFBP3613, *E. persicinus* CFBP3622, *E. rhapontici* CFBP3618, *E. nigrifluens* CFBP3616, and *E. amylovora* CFBP1232

^d *Xanthomonas campestris* pv. *campestris* XCC356, *E. amylovora* Ea1/79, *E. carotovora* subsp. *atroseptica* Eca185, and *E. carotovora* subsp. *carotovora* Ecc499

strains were gram negative, motile, facultatively anaerobic, reacted with phage Φ K9, and elicited the HR on tobacco. Three strains produced a few water-soaked lesions but did not cause wilting of sweet corn seedlings. They were reisolated from the infected plants and apparently were the cause of the lesions. In tests to differentiate *P. agglomerans* and *P. ananas*, all 11 *Pantoea* strains shared similar biochemical characteristics; they produced acid from glycerol, melibiose, meso-inositol, and sorbitol, and they were unable to use malonate. GC-FAME analysis of four representative strains from this group indicated they were *P. ananas*. The remaining 11 strains were a heterogeneous group of yellow-pigmented, HR⁻, gram-negative, and gram-positive bacteria. None of them reacted with the ITS primer sets or were sensitive to phage Φ K9, but several displayed delayed lesion formation on sweet corn seedlings. Five were aerobes and the rest were facultative anaerobes. GC-FAME was only able to identify one strain as *P. agglomerans* and another as *Pseudomonas aureofaciens*.

Identification of *Pantoea stewartii* by PCR with primers from *hrpS*. Two 17-mer primers, HRP1d and HRP3r, were designed to amplify a 0.90-kb fragment from the *hrpS* ORF (bp +21 to +910; Genbank AF282857). The nucleotide sequences were chosen from a region of *hrpS* that is significantly different in the *E. amylovora* (Genbank AF083877) and *P. agglomerans* pv.

gypsophylae (Genbank AF272053) *hrpS* homologs (Fig. 1B).

We tested the HRP primers against 16 known strains of *P. stewartii* from different geographic regions in the United States, 25 strains of other *Erwinia* and *Pantoea* spp., and 1 strain of *X. campestris* pv. *campestris* (Table 2). All of the *P. stewartii* strains tested positive for the 0.90-kb *hrpS* amplicon (Fig. 3), except for one avirulent strain, DC116. None of the strains of *P. agglomerans*, *P. ananas*, *Erwinia* spp., or *X. campestris* pv. *campestris* produced a specific product even when a high concentration of cells was used (5×10^5 CFU/assay), except for *P. agglomerans* pv. *herbicola* Eh7/95. To detect *P. stewartii* by PCR with the HRP primers, the minimal concentration of bacterial cells needed to obtain a visible amplification product was 20 CFU.

Identification of *P. stewartii* by PCR with primers from the *cps* region. To detect the corn pathogen *P. stewartii* by an additional PCR assay, a pair of primers was designed from the *cpsD* and *cpsE* genes, which encode glycosyltransferases for the biosynthesis of stewartan (14). One of the three genes that differ between the *P. stewartii* and *E. amylovora* EPS gene clusters is *cpsD*, and it appears to be unique to *P. stewartii*. Primer CPSL1 is located in the end of *cpsD* and primer CPSR2c located in the middle of *cpsE*; together they amplified a 1.1-kb DNA fragment.

P. stewartii cells were used directly in PCR assays (Table 2). Of 16 known strains of *P. stewartii* isolated from different regions, 15 gave the expected 1.1-kb amplicon. Only DC116, which is nonmucoid and avirulent, was negative. The minimal con-

Fig. 1. Partial nucleotide sequences of **A**, the 16S rRNA and **B**, the *hrpS* regions of *Pantoea stewartii* and other bacteria used for selection of polymerase chain reaction primers. Alignments were done with the sequences of *P. stewartii* DC283, *Erwinia amylovora* Ea1/79, *Escherichia coli* K-12, *Erwinia pyrifoliae* Ep1/96, and *P. agglomerans* pv. *gypsophylae* (accession numbers given in the text). Primers ES16, HRP1d, and HRP3c are indicated by arrows.

Table 3. Analysis of yellow isolates from corn by polymerase chain reaction (PCR), plant assays, and phage typing for identification of *Pantoea stewartii* subsp. *stewartii*

Strain	Path ^b	HR ^c	PCR primers/amplicon ^a	
			IG1/2 (0.29 kb)	16S/IG (0.92 kb)
<i>P. stewartii</i> subsp. <i>stewartii</i>				
DC422, DC424, DC436, DC438, DC440, DC441, DC442	3	+	+	+
<i>P. ananas</i>				
DC131, DC136 ^d	nd	+	—	—
DC415	0.7	+	—	—
DC416, DC421, DC423, DC426, DC435	0	+	—	—
DC417	0.2	+	v	v
DC418	0	+	v	v
DC427	0	+	—	v
DC430	0	+	v	—
DC434 ^e	1.0	+	—	—
<i>P. agglomerans</i> pv. <i>herbicola</i> DC432	1.1	—	—	—
<i>Pseudomonas aureofaciens</i> DC420	0.7	—	v	—
Unidentified, aerobic, gram negative				
DC419, DC425, DC431	0	—	—	—
DC428	0.4	—	v	—
Unidentified, facultative anaerobic, gram negative DC439	0	—	—	—
<i>Curtobacterium</i> spp. DC433	0.6	—	—	—
Unidentified, facultative anaerobic, gram positive				
DC429	0.4	—	—	—
DC444 ^e	0.1 ^f	—	—	—
DC443 ^e	2.2 ^f	—	—	—

^a nd = not done, v = PCR reaction giving variable intensity of bands (0 to 30% of normal).

^b Virulence assays done on sweet corn seedlings by whorl inoculation and rated at 10 days on a 0-to-3 scale where 0 = no symptoms, 1 = a few lesions but no ooze, 2 = many lesions and some ooze, and 3 = coalescing lesions and ooze.

^c Hypersensitive response (HR) on tobacco.

^d Obtained from corn leaves by S. Walker in Missouri in 1976, but later identified as part of this study.

^e These strains were also assayed with the HRP and CPS primers and no amplicon was obtained.

^f Lesion formation is delayed.

centration of bacterial cells needed to obtain a visible PCR fragment was 20 CFU/assay. All other plant-associated *Erwinia* and *Pantoea* spp. listed in Table 2 (27 strains), as well as *X. campestris* pv.

campestris XCC356 and *Pseudomonas syringae* pv. *syringae* 4U, either did not give the 1.1-kb amplicon or else occasionally gave nonspecific DNA bands of a different size when high amounts of

cells ($>10^5$ cells/assay) were used. *Pantoea agglomerans* pv. *herbicola* Eh7/95 gave an amplification product of approximately 1.1 kb, although this strain was negative with the other primers designed to identify *P. stewartii*.

Use of the PCR assays with mixed cultures and plant material. To simulate mixed bacterial cultures that might be obtained when isolating from plants, *P. stewartii* cells (5×10^2 CFU/assay) were mixed with either *Pseudomonas syringae* pv. *syringae* 4U, *X. campestris* pv. *campestris* XCC356, *E. carotovora* subsp. *atroseptica* Eca185, *Pantoea agglomerans* pv. *herbicola* EhNZ, or *E. amylovora* E9 cells (1×10^6 CFU/assay) and the mixtures were used in PCR assays with the CPS primers. In all cases, the 1.1-kb amplicon was detected when *P. stewartii* cells were added, but it was not present in the controls (Fig. 4).

Leaves from healthy and inoculated sweet corn also were assayed for the presence of *P. stewartii*. The tissue suspensions from lesions on infected plants gave the correct amplicon with all four PCR primer pairs, whereas tissue from healthy plants tested negative. The specific amplicon could be visualized even when the aqueous suspension of infected leaf tissue was diluted as much as 1,000-fold. Plating showed that the minimum number of *P. stewartii* cells needed for detection from leaf tissue was 200 CFU.

PFGE assays of *P. stewartii* strains and a comparison with other *Erwinia* and *Pantoea* spp. *Erwinia* and *Pantoea* spp. have a high GC content (50 to 58%); therefore, we chose the restriction endonucleases *SpeI* and *XbaI*, which recognize an AT-rich hexanucleotide sequence, to produce genomic DNA restriction fragments. The *XbaI* restriction digest gave rise to fewer fragments than the *SpeI* digest. Eight strains of *P. stewartii* were examined and these yielded at least 12 *XbaI* bands in the range of 100 to 800 kb (Fig. 5A). Common *XbaI* fragments of 375, 320, 270, 235, 210, and 135 kb were shared by all strains. *P. stewartii* strains were easily distinguished from other *Erwinia* and *Pantoea* spp. by their PFGE fingerprints. A large, 150-kb plasmid was also observed in the PFGE analysis of undigested chromosomal DNA of DC283. This confirmed the result obtained by Coplin et al. (15) that *P. stewartii* possesses large plasmids.

In an *SpeI* digest, *P. stewartii* strains, isolated from different geographic origins at different times, had divergent restriction patterns, except for SW19 and DC145 (Fig. 5B), which also had an identical pattern in *XbaI* digests. These two strains were isolated from different states, 1 year apart. However, the patterns of the nalidixic acid-resistant strain DC283 and its reported parent strain SS104 also were different, suggesting that genetic rearrangements occurred after 24 years in cul-

Fig. 2. Amplification of the 16S/23S rRNA spacer region using primers designed to detect *Pantoea stewartii*. Products have been separated by agarose gel electrophoresis. **A**, ESIG1/ESIG2c primers (0.29 kb) and **B**, ES16/ESIG2c primers (0.92 kb). Lanes 1 to 4, *P. stewartii* DC283, SW2, SS104, and DC117, respectively; lanes 5 to 9, *P. agglomerans* pv. *herbicola* EhA1-81, EhY112-9, EhA1-82, Eh7/95, and Eh8/96, respectively; lane 10, water control; and lane M, 1-kb ladder marker DNA (Life Technologies GIBCO BRL, Gaithersburg, MD).

Fig. 3. Amplification of the *hrpS* gene using primers HRP1d and HRP3r, designed to detect *Pantoea stewartii*. Products have been separated by agarose gel electrophoresis. Lanes 1 to 7, *P. stewartii* strains SW2, SW4, SW5, SW19, DC283, SS104, and DC117, respectively; lanes 8 to 10, *P. agglomerans* pv. *herbicola* strains Eh7/95, Eh8/96, and EhNZ, respectively; lane C, water control; and lane M, 1-kb ladder marker DNA (Life Technologies GIBCO BRL, Gaithersburg, MD).

ture. Three common bands of 145, 230, and 325 kb could be observed among more than 20 fragments from the eight strains. The similarity coefficient among the strains ranged from 60 to 100% based on the PFGE patterns. The *P. agglomerans* pv. *herbicola* strains Eh7/95 and Eh8/96 from corn leaves in Germany showed completely different *Xba*I and *Spe*I patterns

when compared with *P. stewartii* strains and clearly did not belong to this species. Comparison of the PFGE patterns of *P. stewartii* strains with patterns obtained with other species, such as *P. agglomerans*, *P. ananas*, or *E. carotovora*, also revealed distinct patterns after *Xba*I digestion of genomic DNA and subsequent PFGE analysis (*data not shown*). These data

suggest that PFGE analysis is not only useful for differentiation of *P. stewartii* strains, but is also very useful to confirm species identification.

DISCUSSION

In most instances, the symptoms of Stewart's wilt are very distinct and lesions are associated with flea beetle feeding scars. This makes routine diagnosis of this disease in the field rather simple, when followed by the microscopic observation of ooze in the laboratory. However, Stewart's wilt can be confused with Goss' wilt, caused by *Clavibacter michiganensis* subsp. *nebraskensis* and, to complicate matters, some normally saprophytic bacteria, such as *P. ananas*, can cause water-soaked lesions in greenhouse tests. This means that casual diagnosis of Stewart's wilt is not sufficient to meet plant quarantine requirements. It is recommended that (i) quarantine countries survey regularly for Stewart's wilt, (ii) seed production fields be inspected for systemic infection by *P. stewartii*, and (iii) seed from infested fields be tested by ELISA. Currently, an ELISA test (22) is the only accepted means of detection of *P. stewartii* in seed, because PCR assays have not been sufficiently developed and tested. Commercial ELISA kits represent a significant expense in terms of materials and time; therefore, there is a need for a one-step, highly specific PCR assay for *P. stewartii*. PCR assays, such as those we have developed in this study, should be valuable for seed

Fig. 4. Amplification of the *Pantoea stewartii* *cpsD-E* region using primers CPSL1 and CPSR2c in the presence of other plant-associated bacteria. Products have been separated by agarose gel electrophoresis. *P. stewartii* was added at 500 CFU/assay; the other bacteria at 10^6 CFU/assay. Lane 1, *P. stewartii* DC283 with *Pseudomonas syringae* 4U; lane 2, DC283 with *Xanthomonas campestris* pv. *campestris* XCC356; lane 3, DC283 with *Erwinia carotovora* subsp. *atroseptica* Eca185; lane 4, DC283 with *Pantoea agglomerans* pv. *herbicola* EhNZ; lane 5, DC283 with *E. amylovora* E9; lanes 6 to 10 without addition of *P. stewartii*; 4U, XCC356, Eca185, EhNZ, and E9, respectively; lane C, water control; and lane M, 1-kb ladder (Life Technologies GIBCO BRL, Gaithersburg, MD).

Fig. 5. Separation of fragments from **A**, *Xba*I- and **B**, *Spe*I-digested chromosomal DNA of *Pantoea stewartii* strains and other plant-associated bacteria by pulsed field gel electrophoresis. Lanes 1 to 8, *P. stewartii* strains SW2, SW4, SW5, SW19, DC283, SS104, DC117, and DC145, respectively; lanes 9 to 10, *P. agglomerans* pv. *herbicola* Eh7/95 and Eh8/96, respectively; lane M, ladder of phage λ oligomers (New England Biolabs, Beverly MA). Linear ramping times were from 5 to 25 s over 22 h and from 1 to 40 s over 18 h at 5 V/cm.

certification tests, to confirm field and clinic diagnoses, and in epidemiological studies involving both seed and insect vectors.

Preliminary identification of suspected *P. stewartii* isolates from corn plants can be done by pigmentation, gram reaction (KOH test), motility, and oxidation/fermentation tests, followed by a pathogenicity test. However, for quicker and more certain confirmation of species identification, one must resort to metabolic profiling (29), FAME, plasmid profiles (15), 16S rDNA sequencing, or other systems for bacterial identification. The PFGE fingerprinting technique presented in this study is a reliable, but sumptuous, test for confirming the identity of *P. stewartii* isolates. PCR is a fast, specific, and sensitive method and many plant pathology labs are now equipped for it; therefore, this type of assay is greatly needed. The one-step PCR assays reported here are quicker and cheaper to perform than the LCR and nested PCR assays previously reported (4,30) and appear to be just as specific and sensitive. One-step PCR assays also will accelerate detection time and minimize the risk of contamination, which is increased in nested PCR.

In this study, several sets of PCR primers were tested for the detection and identification of *P. stewartii* from mixed and pure cultures of bacteria, as well as plant material. These tests were based on sequences from two EPS genes, an *hrp* gene, and the 16S rRNA ITS region. All of the primers worked well in distinguishing *P. stewartii* from a large collection of related yellow bacteria that are commonly isolated from corn leaves and from strains representing 17 species from the former *Erwinia* group. With one exception, they detected avirulent as well as virulent *P. stewartii* strains. *P. stewartii* strain DC116, which was identified by biochemical tests and GC-FAME, reacted with the ITS primers but not the HRP or CPS primers. This strain was originally weakly virulent and mucoid when it was isolated in 1976. At that time, it may have had a defective *hrp* cluster and then lost part of the *cps* cluster in storage. The primers also worked well with mixed cultures and plant material. The PCR assay detected 20 CFU using cultured cells and 200 CFU from infected plant tissue. This sensitivity compares favorably with that of ELISA (2×10^4 CFU/g of seed; 22) and LCR (1.9×10^3 CFU/g of plant tissue or 1.0×10^4 CFU/ml from a culture; 30). Moreover, interference by plant compounds did not seem to be a problem; the primers were able to detect *P. stewartii* in fresh lesions and gave no false reactions with samples from healthy corn plants or extracts from corn seed. The four primer pairs infrequently gave nonspecific bands with closely related species. It is possible that the stringency of the reaction could be further adjusted to eliminate the

few weak reactions that we got with several strains of *P. ananas* and one of *P. agglomerans* using the ITS primers. PCR therefore appears to be a more convenient and reliable way to identify *P. stewartii* than pathogenicity tests and many deterministic tests.

PFGE analysis of *P. stewartii* revealed many common bands among strains from different geographic regions. The genome of *P. stewartii* strains seems to be highly conserved (similarity from 60 to 100%) based on these common gene fragments. PFGE also easily distinguished *P. stewartii* from other *Pantoea* and *Erwinia* spp. For example, the PFGE pattern of *P. agglomerans* pv. *herbicola* strain Eh7/95 is completely different from that of *P. stewartii*. On the other hand, there was sufficient divergence in the PFGE profiles to differentiate between *P. stewartii* strains from different geographic regions, so that PFGE also may be a useful tool for population genetics and epidemiological studies.

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In the article "Identification of *Pantoea stewartii* subsp. *stewartii* by PCR and Strain Differentiation by PFGE" by David L. Coplin, Doris R. Majerczak, Yongxiang Zhang, Won-Sik Kim, Susanne Jock, and Klaus Geider, pages 304-311, on page 305, column 3, the last sentence should read "After 35 cycles (1.5 to 2 h), the PCR product was separated on a 1% agarose gel (1.5 to 2 h at 1.5 V/cm), stained with ethidium bromide, and photographed under UV light."